Coenzyme A as a central player in cellular and tissue homeostasis

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Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 25 januari 2012
om 12:45 uur

door

Katarzyna Anna Siudeja
geboren op 19 oktober 1982
te Ozimek, Polen

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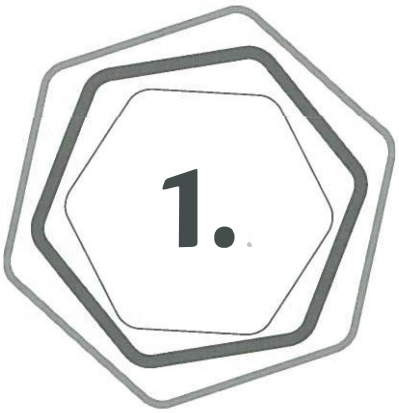
STELLINGEN

behorende bij het proefschrift
Coenzyme A as a central player in cellular and tissue homeostasis
Katarzyna Siudeja

1. The best model of a cat is another cat or, better, the cat itself. *[N. Wiener]*
2. Impaired Coenzyme A biosynthesis alters cell cycle progression *[This thesis]*, thus it cannot be excluded that it affects actively cycling cells (stem cells) in an adult organism. This could influence negatively the plasticity and regenerative capacity of tissues.
3. Reduced levels of Coenzyme A coincide with reduced acetylation levels of histones and tubulin *[This thesis]*, and it is of interest that abnormal acetylation of these "marker" proteins is associated with neurodegenerative conditions.
4. Impaired Coenzyme A metabolism influences actin associated cellular evens, such as the regulation of the cell shape, via a conserved mechanism of cofilin inactivation in response to decreased Coenzyme A levels. *[This thesis]*
5. Our notions of family, population and race may need revising in the age of personal genomics. It is not inconceivable that studies investigating the genetic basis of diseases will reveal people's previously unknown cousins, siblings, half-siblings or even parents. *[A. Chakravarti (2009) Nature]*
6. The prospect of a society that entirely rejects the values of science and expertise is too awful to contemplate. *[H. Collins (2009) Nature]*
7. 7. 2050 years ago Eratosthenes calculated (with an amazing precision) the circumference of the Earth and the tilt of the Earth's axis. Yet, even in the XXI century thousands of people have joined the Flat Earth Society created in 1956 to advocate against the idea of the spherical Globe. *[Wikipedia]* Unbelievable is the power of human ignorance.
8. While living in The Netherlands, you quickly learn to always carry your own sunshine. In fact, on should keep carrying it regardless of the geographical location or the weather forecast.
9. In order to obtain a successful scientist mix gently: a piece of an analytical mind, a large quantity of curiosity, spoonful of passion and determination, and a finishing touch of excellent political skills.
10. Happy is he who gets to know the reasons for things. *[Virgil]*

TABLE OF CONTENTS



1.

Introduction and aim of the thesis

Contents:

Introduction

Coenzyme A – structure and function

The early history of coenzyme A (CoA) research was recapitulated by Fritz Lipmann, a Nobel awarded CoA discoverer. In 1953 The Nobel Prize in Physiology or Medicine was divided equally between Hans Adolf Krebs "for his discovery of the citric acid cycle" and Fritz Albert Lipmann "for his discovery of co-enzyme A and its importance for intermediary metabolism" (http://nobelprize.org/nobel_prizes/medicine/laureates/1953/lipmann-lecture.html). Lipmann ends his Nobel lecture with the following words: "Altogether, in this area, a diversified picture is rapidly developing. There is good reason to hope that in the not too distant future, out of the fair confusion of the present, a clearer understanding will eventually evolve. A new level of complexity seems slowly to unravel and the gap between the biochemical and biological approach further narrows down." Indeed, in the years that followed, detailed studies on essential cellular metabolites, including studies on CoA, have narrowed the gap between biochemistry and physiology.

Coenzyme A is a prerequisite metabolite found in all living organism. It is estimated that CoA is an obligatory cofactor for about 4 % of all known enzymes and it is involved in as many as 100 different reactions of intermediary metabolism [1, 2]. Chemically CoA is derived from vitamin B5 (pantothenic acid), cysteine and ATP (Figure 1). Due to the presence of a thiol group, CoA reacts with carboxylic acids to form thioesters. Thus, CoA functions as a carrier of acyl groups. In fact, most of organic acids require activation to coenzyme A thioesters in order to participate in biochemical reactions [3]. These CoA-activated thioesters, ranging from short to long chain species, take part in numerous cellular processes including tricarboxylic acid cycle (Kreb's cycle, TCA), fatty acid metabolism (lipid synthesis and β-oxidation) and the synthesis of some amino acids. Acetyl-CoA has been proposed to serve, next to ATP and NAD+, as a central small-molecule energetic cell sensor [4]. Numerous acetyltransferases use acetyl-CoA as a sole substrate for the posttranslational protein acetylation and this modification serves as a dynamic switch for major metabolic pathways [5, 6] as well as for regulation of gene function [7, 8]. Additionally to its function as a direct enzymatic cofactor, CoA is also the source of 4'-phosphopantetheine, which serves as a prostetic group of carrier proteins playing roles in fatty acid, polyketide and non-ribosomal peptide synthesis [9].

On the account of the above mentioned functions, CoA and acetyl-CoA appear as major hubs on the complex charts of cellular metabolism. Their crucial role in many interconnecting chemical reactions implies that changes in the free or acetyl-CoA levels may simultaneously disrupt multiple pathways.

Cellular distribution of CoA

In bacteria the intracellular CoA pool varies greatly according to the carbon source used for growth. In E.coli CoA levels are the highest when grown on glucose rich medium, whereas the amount of total CoA is decreased drastically in bacteria grown on mixture of amino acids [10].

Eukaryotic cells contain isolated pools of CoA separated by intracellular membranes. CoA and acetyl-CoA do not cross biological membranes, thus voltage dependant uptake mechanisms exist to support transport between the cytoplasm and organelles [11]. Most of the CoA in cells reside in mitochondria [12]. Mitochondrial CoA is mainly used as a cofactor in TCA cycle and fatty acid β-oxidation and concentrations of free CoA or its thioesters are known to regulate the rates of these processes [13]. The second largest pool of cellular CoA is within the peroxisomes and it plays a major role in β-oxidation of very long chain fatty acids [14]. Compared to mitochondria and peroxisomes, CoA concentrations in the cytoplasm are the lowest. Cytosolic CoA is used to support lipid synthesis, microsomal fatty acid oxidation, protein modification and membrane trafficking. Finally, CoA can be found in the endoplasmic reticulum where the formation of O-acetylated gangliosydes and other sugars takes place [15].

The concentrations of free CoA as well as of individual CoA thioesters are highly important for normal metabolism and their levels are tightly controlled. The transfer of CoA activated acyl chains across organelle membranes (mitochondrial and peroxisomal) is achieved via the carnitine system (reviewed in [16]). This way shuttling of acyl-CoA moieties allows to maintain the cellular CoA pools and CoA availability [17].

Tissue CoA content

The levels of CoA in animal tissues are believed to be regulated in response to various stimuli. The highest and the most fluctuating are the levels of hepatic CoA [18-20]. In the rat liver CoA increases in conditions such as diabetes [21, 22], fasting [20-22], treatment with hypolipidemic drugs [14, 23] or feeding a high fat diet [14, 24]. Among other tissues with high CoA levels are heart, kidney, testis and brain [18]. However, it is not known, if and how, CoA levels are regulated upon different stimuli in these tissues.

On the contrary, low levels of CoA are found in animal tumors, which is in agreement with cancer cells relaying mainly on glycolysis, and to a lesser extent on oxidative phosphorylation and the TCA cycle [25].

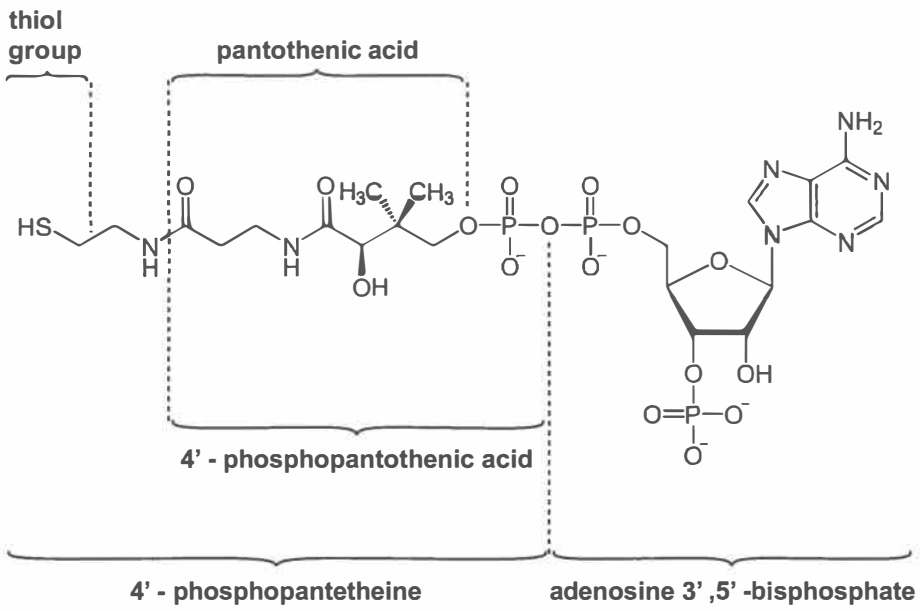


Figure 1. Structure of Coenzyme A

The components from which coenzyme A is synthesized are easily distinguishable: adenine, pantothenic acid and a cysteine-derived thiol group.

Coenzyme A *de novo* biosynthesis

The Coenzyme A *de novo* biosynthesis pathway is highly conserved between various species (reviewed in [2]). The precursor of CoA is vitamin B5 (pantothenic acid), which can be synthesized by plants [26] as well as by most of the bacteria [18]. Animals rely on the dietary supplementation of pantothenate as well as on the supply of pantothenate by the intestinal flora [18, 27]. In fact, due to the abundance of this vitamin in nature, pantothenic acid deficiency in humans has not been reported. On the other hand, it has been shown that dietary pantothenate loading can increase hepatic CoA levels [28].

The first and rate-limiting enzyme of CoA biosynthesis, known as pantothenate kinase or PANK, catalyzes ATP-dependent conversion of pantothenate to 4'-phosphopantothenate (Figure 2). Pantothenate kinase activity is subjected to a feed-back inhibitory regulation by end products of the pathway - CoA and acyl-CoA species. The enzyme shows high homology between species, although some differences between activity, feedback regulation and substrate binding sites among bacterial and eukaryotic PANKs have been described (reviewed in [2]).

In mammals four PANK isoforms, PANK 1-4, are encoded by four distinct genes [29, 30]. The isoforms differ with regard to their patterns of feed-back regulation as well as to their cellular and tissue distribution, which offer a potential possibility for differential regulation of CoA biosynthesis. Nevertheless, the precise roles of each mammalian PANKisoform remain to be elucidated. One isoform, PANK2, is localized to mitochondria in humans [31-33] and is associated to the only known inborn error associated with CoA *de novo* biosynthesis. Mutations in the *PANK2* gene cause hereditary neurodegeneration known as Pantothenate Kinase Associated Neurodegeneration (PKAN) [30]. The disease phenotypes will be addressed in more details in the next section of this chapter. The other PANK isoforms do not possess mitochondrial targeting sequences. The human *PANK2* gene is reported to be transcribed in most tissues, the highest transcripts levels are present in liver. *PANK1* is expressed in heart, liver and kidney, whereas *PANK3* is most abundant in liver tissue. *PANK4* is present in all tissues but highest expressed in muscles [30]. However, PANK4 isoform lacks the essential catalytic glutamate residue present in all PANKs and PANK4 is not enzymatically active when expressed *in vitro* in HEK293T cells [19], suggesting that it does not contribute to the total pantothenate kinase activity of PANK1-3.

In contrast to mice and human, in *Drosophila* there is only one gene encoding pantothenate kinase, *dPANK/fbl*, however, four transcripts of this gene arise from alternative splicing sides. The longest variant (named *dPANK/fbl* E), similarly to human PANK2, has an N-terminal mitochondrial targeting signal and has been shown to reside in mitochondria [34]. Although the enzymatic activity or modes of regulation of the dPANK/Fbl protein have not been experimentally demonstrated, the high degree of sequence homology with PANK proteins of other species strongly supports its annotation [35, 36].

The second step in the CoA biosynthesis pathway consists of ATP dependent condensation of 4'-phosphopantothenate with cysteine catalyzed by 4'-phospshopantothenoylcysteine synthetase (PPCS) (Figure 2). The product of this reaction is further decarboxylated by 4'-phosphopantothenoylcysteine decarboxylase (PPCDC). Both of the above enzymes (in contrast to PANK) are encoded by single gene copies in the human [37] and in the *Drosophila* genome [36]. Subsequently, the AMP moiety from ATP is added to form dephospho-CoA, which is then phosphorylated to form the final product – Coenzyme A. In mammals the last two steps are catalyzed by the bifunctional 4'-phosphopantetheine adenylyltransferase/dephospho-CoA kinase (PPAT/DPCK), also known simply as CoA synthase.

The precise intracellular localization of the entire CoA biosynthesis pathway has long been under debate. Experimental data show that CoA synthase (the last enzyme of the pathway) is localized on the cytoplasmic side of the outer mitochondrial membrane [38, 39]. The two upstream enzymes, PPCS and PPCDC, are predicted to reside

exclusively in the cytoplasm. Finally, the localization of PANK is cytosolic for all the isoforms except for the mitochondrial PANK2, which is now believed to reside in the intramembrane space (IMS) rather than mitochondrial matrix [40-42]. The cytosolic concentrations of CoA species equilibrate with the IMS, due to the fact that the outer mitochondrial membrane is highly permeable to small molecules with molecular mass below 5 kDa [43]. As proposed by Leonardi et al., the benefit arising from PANK2 localization in the IMS is the additional level of regulation of PANK activity via the carnitine system, which shuttles acyl groups through the mitochondrial membrane and which is required for mitochondrial β-oxidation. PANK2 is activated by the long chain acylcarnitine (palmitoylcarnitine). This activation counteracts the negative feed-back regulation of PANK2 activity by acetyl-CoA [42]. Thus, the IMS localization of PANK2 places it in the ideal subcellular localization to sense the levels of long-chain acylcarnitine and the status of mitochondrial β-oxidation, and to adjust rates of cytosolic production of CoA accordingly.

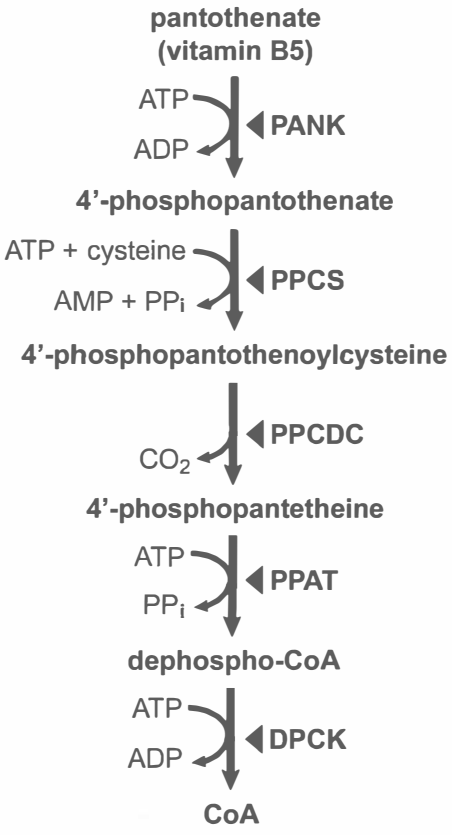


Figure 2. Coenzyme A *de novo* biosynthesis pathway.

Pantothenic acid (vitamin B5) is phosphorylated by pantothenate kinase (PANK) in the first and rate limiting step of CoA biosynthesis. Next, cysteine is incorporated by 4'-phospshopantothenoylcysteine synthetase (PPCS), followed by decarboxylation catalyzed by 4'-phospshopantothenoylcysteine decarboxylase (PPCDC). Further, the adenylyl group is added by 4'-phosphopantetheine adenylyltransferase (PPAT). In the finalt step dephospho-CoA is phosphorylated in ATP dependent reaction catalyzed by dephospho-CoA kinase (DPCK).

Consequences of impaired CoA biosynthesis

CoA deficiency in bacteria

The first studies addressing the consequences of disturbed CoA metabolism were focused on *Escherichia coli* temperature sensitive strains. Decrease in the cellular CoA levels in these bacteria results in growth delay, which directly illustrates the importance of CoA to cell growth [44, 45]. The onset of growth stasis is due to the inability to synthesize amino acids and proteins. Additionally, CoA depletion in *E. coli* causes a change in the composition of membrane phospholipids (saturated/unsaturated fatty acids ratio is affected), although fatty acid and phospholipid production is not completely arrested [45]. These observations show that, at least in *E. coli*, restricted supply of CoA is more deleterious to protein synthesis than to fatty acid production. However, the studies based on prokaryots cannot be easily translated to eukaryotic and/or multicellular models.

Pantothenate kinase-associated neurodegeneration (PKAN)

For about two decades potential consequences of decreased CoA levels were not further addressed. The CoA biosynthesis pathway has only gained renewed attention after the discovery that mutations in the human *PANK2* gene cause the severe neurodegeneration PKAN [30]. This discovery revealed an unexpected link between CoA metabolism and neurological function. PKAN patients suffer from progressive impairment of movement, speech and cognition [46, 47]. It has to be stressed that it is not currently known if these patients show decreased levels of CoA in any tissue. Some of the clinically identified PANK2 mutations lead to an inactive protein, but others still give rise to a protein with enzymatic activity [33, 48]. Thus, the detailed pathogenesis of PKAN is unknown. Two hypotheses have been proposed to explain the pathophysiology of this devastating disease. The first explanation is that the syndrome could result simply from the product deficit – CoA depletion. In this case, tissues with the highest CoA demand (showing the highest metabolic rates) would be most severely affected. Secondly, the pathogenesis can be explained by a mechanism in which accumulation of specific metabolites occurs. Normally, cysteine is incorporated in the second step of the CoA biosynthesis pathway (downstream of PANK activity). Thus, in PKAN patients a block in this step would lead to cysteine accumulation [49]. The toxicity of cysteine is well documented [50, 51]. In the presence of accumulated iron, which is also a characteristic of the PKAN patient's brain, cysteine undergoes rapid autooxidation resulting in production of free radicals (reactive oxygen species, ROS). Cysteine and ROS accumulation, as well as oxidative damage in general, are associated with a range of diseases, including neurodegeneration such as Alzheimer's or Parkinson's disease [52-54]. Thus, it is tempting to speculate that these factors could also play an important role in PKAN pathogenesis. Nevertheless, further studies are needed to distinguish between the primary triggers of PKAN syndrome and the secondary consequences of the disease.

Currently there is no cure for PKAN. Pharmacological treatment is only focused on relieving the symptoms. In some cases treatment with vitamins and antioxidants (like pantothenate or Coenzyme Q) has proved beneficial [55], however these results have not be confirmed in large scale studies. Because of high iron deposits in patients' brains, iron chelating agents are currently being tested in clinical trials. Animal models of PKAN have recently been created, to facilitate the discovery and testing of potential treatments.

Mouse models of impaired PANK activity

In an attempt to enhance understanding of the PKAN disease and to test possible therapies, *PANK2* knock-out mice were generated. Mice lacking PANK2 show growth retardation (decrease in body mass), progressive retinal degeneration and male infertility due to azoospermia. Although the retinal degeneration in these mice can provide a model to study retinitis pigmentosa observed in PKAN patients, the *PANK2* mouse mutant failed to exhibit movement disorders or brain pathology, characteristics associated with the disease [56]. CoA levels in tissues of *PANK2* mutant mice are not known, neither is the total pantothenate kinase activity. Thus, it is unclear whether other mouse PANK isoforms compensate for the PANK2 knock-out. Additinally, it needs to be stressed that PANK2 knock-down in mice may not reflect precisely PANK2-deficiency in humans. Experimental data suggest that mouse PANK2, in contrast to human PANK2, doesn't possess a mitochondria localization signal and is, in fact, localized to the cytoplasm. Furthermore, PANK2 expression levels are much higher in human brain than in mouse brain [41]. These discrepancies could explain why *PANK2* knock-out mice are most probably not a suitable genetic model to understand the pathophysiology of PKAN.

A more general insight in CoA homeostasis on the organism level was recently gained after the generation of *PANK1* knock-out mice. The PANK1 isoform is expressed in liver and *PANK1* mutant mice were used to determine whether a proper regulation of CoA levels was critical to liver function. *PANK1-/-* mice have decreased total pantothenate kinase activity in liver and decreased liver CoA levels, suggesting that other PANK isoforms do not compensate for the loss of PANK1. However *PANK1-/-* mice do not exhibit gross defects in anatomy or behavior. In contrast, these mice are not fully able to adapt to metabolic stress induced by fasting [20]. Upon fasting CoA deficient livers have low rates of fatty acid oxidation, which is associated with abnormally high accumulation of long chain acyl-CoAs and acyl-carnitines. *PANK1-/-* mice become hypoglycemic during a period of fasting due to impaired gluconeogenesis. In short, PANK1 expression and activity is essential to maintain hepatic CoA levels and to support the metabolic changes during the transition from the fed to the fasted state.

Further, non-genetic approaches have also been taken to study the consequences of impaired CoA biosynthesis in mice. Animals kept on a pantothenate deficient diet

manifested not only decreased body weight and azoospermia (similar to *PANK2* knockout mice), but also movement disorders and signs of dystonia [57], suggesting that pantothenate deprivation provides a useful phenocopy of the human PKAN disorder. Nevertheless, despite the neurological presentation, these mice showed no evidence of brain iron accumulation (the hallmark of PKAN). Movement disorders caused by pantothenate deficiency were observed also in other animals, including dogs, calves, pigs and monkeys [58-60]. Further, CoA deficiency in mice has recently been induced by administration of hopantenate (HoPan), a methylated pantothenate analog which inhibits all enzymatically active mammalian PANK isoforms (PANK1-3) [19]. HoPan dramatically reduced liver CoA levels and the mice became hypoglycemic with fatty liver and abnormal mitochondrial morphology. Metabolic profiling revealed an increase in acylcarnitines, proving a role of the carnitine system in buffering CoA levels. Additionally, HoPan triggered significant changes in hepatic gene expression, including increased expression of thioesterases which cleave acyl-CoAs to free CoA, and increased expression of pyruvate dehydrogenase kinase 1, which prevents the conversion of CoA to acetyl-CoA via the glycolytic flux. All together, this interesting study clearly shows the importance of CoA metabolism especially in hepatic tissue, as well as the first view of the cascade of compensatory changes (on the metabolomic and gene expression level) generated by CoA deficiency and demonstrated that those are directed towards preservation of the free (non-estrified) CoA concentration.

Drosophila as a model to study impaired CoA metabolism

As an alternative to studies in mice, *Drosophila* can also be used to study the consequences of impaired CoA metabolism. *Drosophila melanogaster* is an excellent model organism because of its powerful genetic techniques, short lifespan and relative simplicity of the organism. At the same time, more than 50% of all fly proteins have mammalian analogs and many metabolic pathways are conserved in both men and flies [61, 62]. Thus, *Drosophila* has been successfully used as model to study metabolic defects as well as neurodegenerative diseases [63, 64].

The *Drosophila* ortholog of PANK, *dPANK/fumble*, was identified in a screen for genes involved in male sterility. The *dPANK/fbl* gene encodes the sole *Drosophila* pantothenate kinase and it is essential for flies because excision of the major portion of the fumble gene results in pupal neuroblast abnormalities and lethality [35]. Male sterility of *dPANK/fbl* hypomorphs (flies with reduced expression of dPANK/Fbl) has been studied in detail. Interestingly, defects in cell divisions are described in mutant larval neuroblasts and mutant testis. The cells exhibit aberrant mitoses and meioses with abnormalities in chromosome segregation and impaired formation of the contractile ring. This coincides with disturbed F-actin dynamics during cell divisions. Additionally, the same study shows that during mitosis the dPANK/Fumble protein localizes around the spindle in metaphase, at the cleavage furrow in anaphase and near the spindle midbody dur-

ing telophase. This interesting colocalization of dPANK/Fbl with the mitotic apparatus suggests additional active roles for this CoA biosynthesis enzyme in cellular homeostasis.

In addition to male and female sterility, *dPANK/fbl* mutants are characterized by a neurodegenerative phenotype. Moreover, not only *dPANK/fbl Drosophila* mutants but also dPPCS and dPPAT-DPCK (downstream enzymes of the CoA *de novo* biosynthesis) are neurologically impaired, show a reduced lifespan, a high sensitivity to ROS, an impaired lipid homeostasis, increased levels of DNA damage and hypersensitivity to DNA damaging agents [36]. It is currently not clear why defective CoA biosynthesis induces such a pleiomorphic phenotype in fruit flies. Nevertheless, it appears that the fruit fly model recapitulates several symptoms of the human PKAN syndrome. Consequently *Drosophila* PANK/fbl mutants can be used as a model to study PKAN, as well as a tool to study general consequences of impaired CoA homeostasis.

Aim and Outline of the Thesis

At the start of this research project the *Drosophila* model for Coenzyme A deficiency had been established and initially characterized [36]. In the course of this thesis we further explored this model to gain more inside into the physiological and molecular consequences of disturbed *de novo* biosynthesis of CoA. Thus, this research is directed towards two major goals: (1) understanding the role of CoA in the maintenance of cellular and tissue homeostasis, and (2) extending the fundamental knowledge behind the pantothenate kinase-associated neurodegeneration and providing new clues for potential treatments of this disease.

Chapter 2: The use of *Drosophila* cultured cells to study cell cycle checkpoints

Among the advantages of using *Drosophila in vitro* studies are the ease of maintaining fly cells in culture and the very high efficiency of RNAi-mediated gene knock-down. In this methodological chapter we provide an overview of *Drosophila* cell culturing techniques together with detailed protocols for RNAi experiments. Further, we show how cultured cells can be used for cell cycle studies, with a particular focus on inducing DNA damage and cell cycle checkpoints.

Chapter 3: Impaired cell cycle progression in response to decreased Coenzyme A levels in *Drosophila* S2 cells

In Chapter 3 we use some of the methods introduced in Chapter 2, as well as other techniques, to investigate how impaired CoA biosynthesis affects the proliferation and cell cycle progression of *Drosophila* cultured cells. We show that RNAi-mediated knock-down of dPANK/Fbl reduces cell proliferation, delays progression of cells through G2 phase of the cell cycle and delays entry into mitosis. Thus, we show that hindered CoA metabolism has an impact on various specific cell cycle events and we discuss the potential molecular mechanism behind these observations.

Chapter 4: Pantethine rescues a *Drosophila* model for pantothenate kinase-associated neurodegeneration

In this chapter we use *in vitro* RNAi knock-down as well as *dPANK/fbl* mutant flies to show that decreasing the levels of pantothenate kinase in flies leads to a reduction in total CoA levels. This is further associated with mitochondrial dysfunction, neurological impairment and reduced lifespan. Additionally, we identify pantethine as a compound able to rescue CoA levels and mutant phenotypes upon feeding. Hence, the evidence is shown for a new, PANK independent, CoA generating pathway. Finally, pantethine is also shown to be protective in mammalian cells in which hPANK2 is downregulated. It is discussed how these results can be at the basis of a possible treatment for PKAN.

Chapter 5: Impaired Coenzyme A metabolism affects histone and tubulin acetylation in *Drosophila* and human cell models of pantothenate kinase-associated neurodegeneration

CoA serves as a carrier of acetyl groups and one of the functions of acetyl-CoA in the cell is its participation in post-translational protein acetylation. Although this protein modification is widely studied, the metabolic pathways necessary to support acetylation are poorly understood. In this chapter we use RNAi and biochemical approaches to show that decreased CoA levels lead to impaired acetylation of specific proteins (namely histones and tubulin). Additionally, we show that decreased acetylation underlies the impaired DNA damage responses, decreased survival and impaired locomotor function in a *dPANK/fbl* deficient background. Restoration of normal acetylation levels with deacetylase inhibitors partially rescues these apparent phenotypes.

Chapter 6: Hyperphosphorylation of actin severing protein cofilin in response to impaired Coenzyme A metabolism in *Drosophila* S2 cells and during neuronal differentiation *in vitro*

In this chapter we study the molecular mechanism underlying actin remodeling defects in *dPANK/fbl* mutant background. We show that interfering with PANK activity in *Drosophila* as well as human neuronal cells results in increased phosporylation of cofilin – an actin regulatory protein. Hyperphosphorylation of cofilin in the cells with impaired CoA metabolism coincides with abnormal morphology and abnormal F-actin organization. Furthermore, we show that blocking PANK activity reduces the ability of human cells to form neurites in culture – a process that is strongly dependent on actin remodeling. Thus, this chapter shows the link between CoA metabolism and regulation of actin dynamics, and it speculates on the physiological consequences of actin abnormalities in neuronal function.

Chapter 7: Summarizing discussion and perspectives

The results presented in this thesis elucidate that impaired CoA metabolism has far-reaching (though yet not fully appreciated) consequences for cellular and tissue homeostasis. In this chapter we summarize our findings and discuss perspectives for the future research. We stress the connection between different cellular events affected upon PANK deficiency, which further underscores a central role of CoA on the crossroads of metabolism.

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Studying cell cycle checkpoints using *Drosophila* cultured cells

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Methods Mol Biol. 2011;782:59-73

Abstract

Drosophila cell lines are valuable tools to study a number of cellular processes, including DNA damage responses and cell cycle checkpoint control. Using an *in vitro* system instead of a whole organism has two main advantages: it saves time and simple and effective molecular techniques are available. It has been shown that *Drosophila* cells, similarly to mammalian cells, display cell cycle checkpoint pathways required to survive DNA damaging events [1, 2]. Moreover, a number of proteins involved in checkpoint and cell cycle control in mammals are highly conserved among different species, including *Drosophila* [1-5]. Because of straightforward and highly efficient methods to down-regulate specific transcripts in *Drosophila* cells, these cells are an excellent system for genome-wide RNA interference (RNAi) screens. Thus, the following methods, assays and techniques: *Drosophila* cell culture, RNAi, introducing DNA damaging events, determination of cell cycle arrest, and determination of cell cycle distributions described here may well be applied to identifying new players in checkpoint mechanisms and will be helpful to investigate the function of these new players in detail. Results obtained with studies using *in vitro* systems can subsequently be extended to studies in the complete organism as described in the chapters provided by the Su laboratory and the Takada laboratory.

Introduction

Similar to other organisms, *Drosophila* cells respond to DNA insults via organized and tightly regulated pathways. Checkpoint activation, cell death, and compensatory proliferation were all shown to significantly contribute to the survival of *Drosophila* whole organisms after the exposure to DNA damage inducing agents [1, 6]. Cultured *Drosophila* cells can be applied to study some, although not all, of these responses. *Drosophila* Schneider's cells (commonly known as S2 cells) [7] are often used for these type of studies. Although cell death and apoptosis are difficult to detect in *Drosophila* cells following exposure to DNA damaging agents, it has been proven that S2 cells delay entry into mitosis and display a G2/M checkpoint arrest after exposure to DNA damaging events and compounds such as ionizing radiation or hydroxyurea [1].

The advantages of using an *in vitro* system are listed below:

1. Cell lines are homogenous, easy to handle, and show stable behavior over time when good culturing conditions are applied.
2. The treatment of cell cultures with DNA damage inducing agents (chemicals, ionizing radiation) is easier compared to the intact organism. This ensures an equal exposure and response of the whole cell population, which can additionally be assayed with the use of relatively straightforward tests and reliable quantification methods.
3. It is straightforward and relatively cheap to perform RNA interference (RNAi) experiments in *Drosophila* cells. RNAi is highly effective in these cells and can be applied to analyze consequences of down-regulation of specific checkpoint proteins or can be useful to identify new players involved in checkpoint function.

In this chapter we first introduce the basic techniques in culturing *Drosophila* cells. Handling insect cells follows, to a large extend, the standard protocols developed for mammalian cell lines. Up to date a number of *Drosophila* cell lines have been established. Initially, *Drosophila* cell lines were derived from spontaneously immortalized cells obtained from mechanically dissociated embryos. Among these lines, S2 and Kc cells [7, 8] are currently the best characterized and the most commonly used cell lines by *Drosophila* researchers.

Second, we describe the methods to induce a checkpoint response in *Drosophila* cell cultures. A number of DNA damaging insults can be used in *Drosophila* cells. We and others have been successfully using hydroxyurea (HU) or ionizing radiation (IR) to investigate checkpoint function and cell survival after the induction of DNA damage [1, 2, 9]. Both HU and IR introduce a cell cycle arrest in wild-type cells. HU is a DNA replication inhibitor that decreases the pool of dNTPs, resulting in stalled replication forks [10]. Ionizing radiation, on the other hand, damages DNA by direct deposition of energy, as well as producing hydroxyl radicals that attack the DNA. IR induces multiple forms of DNA damage including double-strand breaks, which are considered to be the most lethal form of DNA damage [11, 12]. After exposure to DNA damaging agents wild-type cells activate a cell cycle checkpoint pathway and a decreased fraction of cells which enter mitosis is observed (Figure 1). Mitotic cells can be stained using an antibody against mitosis specific phosphorylated histone 3 [13]. The amount of cells in mitosis can then be determined using immunofluorescence techniques: fluorescence microscopy or flow cytometry. Additionally, cell cycle distribution can also be studied in *Drosophila* cells with the use of a well known technique of DNA content measurements using propidium iodide staining. Finally, given that *Drosophila* cell lines are especially useful for RNA interference (RNAi) mediated down-regulation of specific genes, we will also include the protocols for designing and performing an RNAi knockdown experiment. These will be useful for all the researchers interested in the effect of the down-regulation of a specific protein on cell cycle checkpoint functioning. RNAi has already been widely used in *Drosophila* cells to dissect signaling pathways and cellular processes [14-18]. In *Drosophila* long double-stranded RNA molecules (dsRNA) can be introduced into the cells without triggering an interferon response and without causing other side effects that are commonly observed in mammalian systems [19-21]. dsRNA molecules taken up by the cells are cleaved by the cellular RNAi machinery and a pool of gene specific short silencing RNAs is generated, resulting in a highly efficient down-regulation of the target mRNA. The RNAi technique applied to cultured *Drosophila* cells has already been proven to be useful in studying, among others, the involvement of *Drosophila* checkpoint kinases, Chk1 homologue grapes (grp/Dchk1) and Chk2 homologue DmChk2, in cell cycle progression after exposure to DNA damaging insults [1]. Down-regulation of a specific gene product involved in cell cycle checkpoint function will result in an increased fraction of mitotic cells after introducing DNA damage to the cells. The above techniques were used to show that in *Drosophila* cells grp/Dchk1 is required for G2/M checkpoint activation, whereas Dmnk/DChk2 is dispensable [1]. Thus, RNAi knock-down of grp/Dchk1 can serve as a control for checkpoint studies in studies to identify new potential genes involved in DNA damage response. For a fast and reliable data analysis the protocols provided here were in part optimized for flow cytometry analysis. Cells fixed and stained according to the protocols provided in this chapter can be analyzed with the use of standard protocols available for a range of cytometers.

Many research centers are equipped with a dedicated cytometry facility providing technical support and knowledge. Detailed protocols for the acquisition and analysis of the flow cytometry data exceed the limits of this chapter. A comprehensive methodology of the flow cytometry of *Drosophila* cells has been described elsewhere [22]. In summary, we provide an easy to follow strategy to down-regulate a specific gene of interest in *Drosophila* cultured cells and to investigate whether this down-regulation affects cell cycle distribution and/or checkpoint functioning.

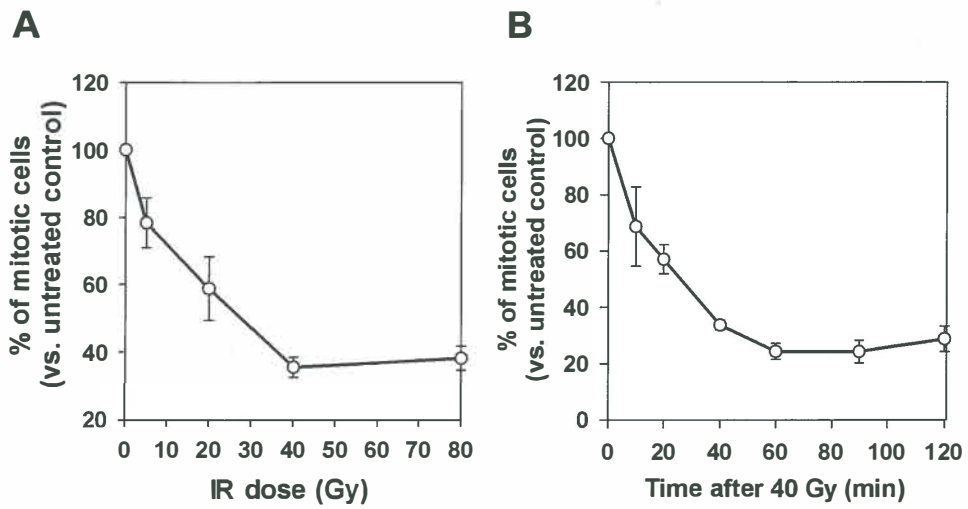

Figure 1. G2/M checkpoint response in wild-type *Drosophila* S2 cells.

Drosophila S2 cells exposed to ionizing radiation (IR) were stained for mitosis specific phosphorylated histone 3 and the percentage of mitotic cells was determined by flow cytometry. A, Dose–response curve. Cells were exposed to increasing doses of IR, allowed to recover for 45 min, and the mitotic index was determined. B, Time kinetics of the mitotic arrest. S2 cells were exposed to 40 Gy of IR, allowed to recover for the indicated times, and the mitotic index was determined.

Materials

Cell culture

1. Schneider's *Drosophila* Medium with L-Glutamine (Invitrogene) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco/Invitrogen or other suppliers, see Note 1), 100 U/ml penicillin and 100 U/ml streptomycin (various suppliers).
2. Phosphate buffer saline (PBS), pH 7,4 (various suppliers)
3. 0.25% Trypsin with 1mM EDTA (Invitrogen)
4. 0,4% Trypan Blue solution (Sigma) and hemocytometer for counting the cells
5. Tissue culture flask, multi-well plates, disposable pipettes.

RNAi

1. PCR reaction: 5 mM oligonucleotides (see Methods for guidelines to design primers), Platinum Blue PCR SuperMix (Invitrogen), PCR template (cDNA)
2. PCR Purification Kit (Qiagen)
3. In vitro transcription: MEGAscript RNAi Kit (Ambion, Catalog #1626)
4. Agarose gel electrophoresis: 0,8 - 1 % agarose in TAE buffer (TAE : 40mM Tris-acetate, 1mM EDTA), 0,5 μg/ml ethidium bromide (from 10mg/ml stock, Invitrogen)
5. Schneider's *Drosophila* Medium with L-Glutamine (Invitrogen) without FBS (see Note 2)

DNA damaging agents

1. Hydroxyurea (HU, Sigma) HU is toxic and should be handled with extra care. Working solution of HU should be prepared fresh for every experiment.
2. Cesium-137 source IBL 637 irradiator (CIS Bio-Internationl) or other source of ionizing radiation (X-rays, gamma-rays).

Immunolabeling of cells for flow cytometry

1. PBS for washing steps.
2. 3,7 % Formaldehyde in PBS. Dilute always freshly before use from commercially available 37 % stock .
3. Methanol (absolute)
4. Incubation buffer: 1% bovine serum albumin (BSA) in PBS (Solution should be stored at 4°C).

5. Primary antibody: polyclonal rabbit anti-phospho-histone H3 (Cell Signaling, see Note 3)
6. Fluorophore conjugated secondary antibody: Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes/Invitrogen, see Note 3)
7. Round bottom 6 ml conical tubes suitable for a cytometer.

Immunolabeling of cells for fluorescent microscopy

1. Poly-L-Lysine coated cover slips. Cover the slips with sterile 0,001 % poly-L-lysine (Sigma) solution and incubate for 10 minutes. Wash the cover slips 3 times with water and allow to dry.
2. PBS for washing steps.
3. 0,1 % Tween in PBS (PBST)
4. 3,7 % Formaldehyde in PBS. Dilute always freshly before use from commercially available 37% stock.
5. 0,2 % Triton X-100 in PBS
6. 3 % BSA in PBS (Solution should be stored at 4°C).
7. Primary antibody: polyclonal rabbit anti-phospho-histone H3 (Cell Signaling, see Note 3)
8. Fluorophore conjugated secondary antibody: Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes/Invitrogen, see Note 3)
9. 0,2 µg/mL DAPI in PBS (Invitrogen)
10. Citifluor mounting medium (Agar Scientific or other suppliers)

DNA staining

1. PBS for washing steps.
2. 80% ethanol:aceton (1:1)
3. DNase free RNaseA (make 10 mg/ml RNaseA stock solution in water and boil for 10 minutes to destroy DNase activity; store at - 20°C in aliquots)
4. Propidium Iodide: 25 µg/mL solution in PBS (solution should be kept in the dark, at 4°C)
5. Round bottom 6 ml conical tubes suitable for cytometer

Methods

Cell culture

Choosing a cell line to study

All *Drosophila* cell lines, together with their descriptions, are available through the *Drosophila* Genomics Resource Center (https://dgrc.cgb.indiana.edu/cells/). The collection currently includes 108 lines. In general, cells of an embryonic origin are easy to handle, fast-growing and very convenient for RNAi experiments. The collection includes also a range of cell lines derived from the central nervous system (CNS) or imaginal discs. These lines are characterized by the presence of tissue specific markers and often posses a distinct morphology but, at the same time, are usually more demanding in culture. The protocols described below were developed mainly for Schneider's cells (S2 cells), nevertheless they may as well be applicable to other cell lines with similar characteristics (like Kc cells).

Guidelines for maintaining *Drosophila* cells in culture

1. Commonly used fast-growing cell lines, like S2 or Kc, should be maintained by splitting the cells twice a week in a dilution 1/10 to 1/20. Other *Drosophila* cell lines can be sensitive to low cell density and should only be diluted 4-6 fold at each passage.
2. Cell viability should be assayed by a Trypan Blue exclusion test [23]. Mix the cell suspension with Trypan Blue solution in 1:1 ratio. Incubate the cells with the Trypan Blue for 5 minutes and transfer the cells in solution to the chambers of a hemocytometer. Cells excluding Trypan Blue are viable, cells containing the Trypan Blue dye are considered to be not-viable.
3. Most *Drosophila* cells adhere loosely to the culturing surfaces and therefore can be easily detached by pipetting the medium over the cell layer. For more strongly adhered cells, trypsin-EDTA solution should be used in order to detach the cells.
4. *Drosophila* cells are grown in a cell culture incubator without CO2 at temperatures ranging from 22°C to 25°C. Culturing at 25°C may be beneficial in reducing the probability of yeast contaminations, since yeast cells prefer to grow at lower temperatures.

RNAi in *Drosophila* cells

dsRNA can easily be obtained in large amounts in a two-step procedure. First a gene-specific DNA template is created by a PCR reaction. Subsequently, the PCR product will be used for an *in vitro* transcription reaction to generate dsRNA (Figure 2). Using a simple transfection protocol, *Drosophila* cells take up dsRNA and no expensive transfection agents are required.

Generating a DNA template for *in vitro* transcription

1. Designing primers. For *in vitro* transcription a gene-specific transcription template with T7 RNA polymerase promoter overhangs is needed. Templates of 300 - 800 bp, with the least possible homology to other targets, should be used (see Note 4). Design the forward and reverse primers such that the 18 - 24 gene specific sequence is proceeded by the T7 promoter sequence: 5' - TAATACGACTCACTATAGGGA - gene specific - 3' (the underlined G will be the transcription start site). As a positive control for cell cycle checkpoint studies, a gene specific transcription template can be generated to down regulate grp/Dchk1. For this the following T7-primers should be used: TAATACGACTCACTATAGGGATGTGCGTGTGTGTGCCG and TAATACGACTCACTATAGGGAGGATATGCTTATCCTG (corresponding to the 493 - 1200 bp fragment of the GeneBank sequence with the accession number: AF057041)
2. Amplify the template with a PCR reaction. In a PCR tube mix 2 µL of 5mM solution of each primer, 50ng of cDNA and Platinum Blue PCR SuperMix to a final volume of 100 µL. For cycles 1-5 use an annealing temperature by calculating the Tm of the sequence specific parts of the primers (Ta1). For cycles 6-30, the annealing temperature should be calculated based on the Tm of the entire PCR primers (Ta2). Set the thermocycler and run the amplification reaction: 94°C for 30 sec, Ta1 for 30 sec (cycle 1-5), Ta2 for 30 sec (cycle 6-30), 72°C for 1minute.
3. Analyze 5 µL of the reaction by agarose gel electrophoresis to verify the product yield. The product should appear as a sharp single band of an expected size.
4. Purify the PCR product using a PCR purification kit (Qiagen) and elute the DNA in RNase free water.
5. Determine the concentration of the PCR product by measuring the A260 absorbance. The following formula can be used for calculating the DNA concentration: DNA [µg/mL] = A260 x 50 x dilution factor.
6. A clean PCR product of a concentration of at least 0,1 µg/µL should be obtained at this step (see Note 5)

Generating dsRNA

1. Perform an *in vitro* transcription reaction with the use of the MEGAScript RNAi Kit (Ambion) according to manufacturer's instructions. Use 1 µg of DNA template to ensure a high transcription yield. Incubate the transcription reaction at 37°C overnight.
2. Check 0,5 µL of the transcription reaction on an agarose gel. At this step the product is usually visible as a smear and not as a distinct band (lane 2, Figure 2B).
3. Proceed with nuclease digestion and purification of dsRNA according to the protocol of the kit.
4. Elute purified dsRNA from the column with pre-warmed Elution Buffer (two times 50 µL).
5. Determine the concentration of dsRNA by measuring the A260 absorbance. Dilute dsRNA 20-100 times in nuclease free water or Elution Buffer. Use the following formula to calculate dsRNA concentration: dsRNA [µg/mL] = A260 x 40 x dilution factor.
6. Check 0,5 µL of eluted dsRNA on an agarose gel. At this step the product should be visible as a distinct band (lane 3, Figure 2B).

RNAi "bathing" technique in *Drosophila* cells

1. Use only healthy, exponentially growing cultures, with a viability of more than 97%.
2. Suspend the cells, transfer them to a conical tube, count and spin the cells down for 5 minutes at 1000 rpm. Resuspend the cells in serum free medium with glutamine to a density of 1 x 106 cells/mL.
3. Transfer 1 mL of cell suspension to each 35 mm dish (or to each well of a 6-well plate).
4. Immediately add 5-15 µg of dsRNA (see Note 6 and Table 1) and mix by gently rocking the dish (plate) back and forth.
5. Incubate the cells with dsRNA for 1 hour.
6. Add 2 mL of complete Schneider's S2 medium to the cells.
7. Test the efficiency of RNAi on the protein or RNA level after 48 - 72 hours.
8. If examination of the cells at later time points is required, split the cells into the density of 0,5 x 106/mL and continue the culture until days 4-9 (see Note 7).

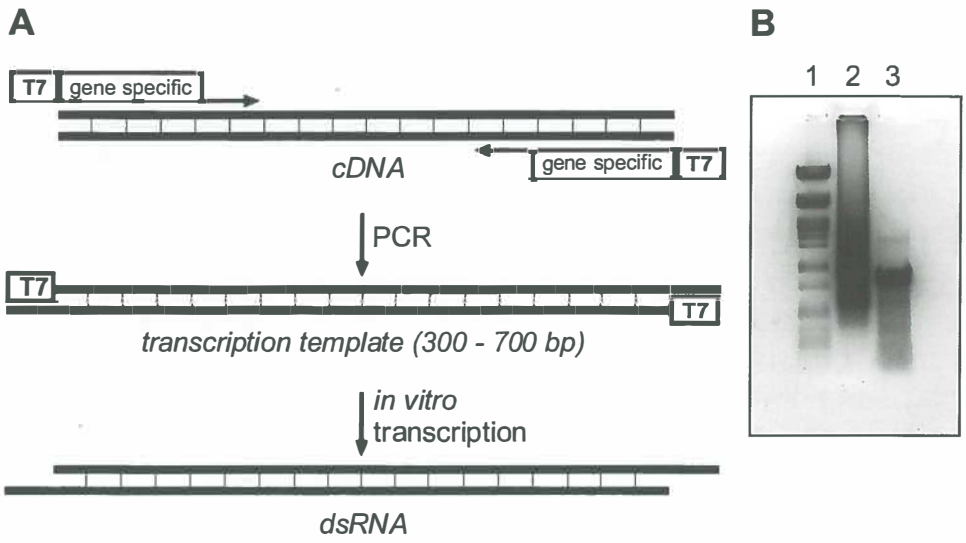

Figure 2. Generation of long dsRNA for RNAi in *Drosophila* cells.

A, Double-stranded DNA template is obtained by standard PCR reaction with gene specific primers flanked with T7 promoter sites. The template is used for an *in vitro* transcription to produce dsRNA. B, An agarose gel illustrating the steps of generating dsRNA: 1 – DNA Ladder; 2 – 1 µL of an *in vitro* transcription reaction; 3 – purified dsRNA of approximately 700 bp.

Exposing cells to DNA damaging insults

In general *Drosophila* cells tend to be more resistant to IR than most commonly used mammalian cells. We routinely use doses of 10 – 50 mM HU in cell culture medium for 6 – 12 hours and an exposing dose of 20 – 160 Gy of radiation.

1. Resuspend the cells in culture medium, count the cells and determine the cell viability as described under 3.1.2. Only exponentially growing cultures, with a viability of more than 97% should be used for an experiment.
2. Seed the cells to a density of 1 x 106/mL in tissue culture dishes or 15 mL conical tubes.
3. Allow the cells to settle down for 30 minutes under standard growth conditions.
4. Add HU to the medium or expose the cells to ionizing radiation. Incubate the cells with HU containing medium for 6 -12 hours and replace the medium with a fresh standard medium.
5. Return the cells to the incubator for a recovery period of a desired time.

Immunolabeling of mitotic cells - flow cytometry

For determining the mitotic index of cells after exposure to checkpoint inducing agents we recommend staining of mitotic chromosomes in formaldehyde fixed cells. The antibody against mitosis-specific phosphorylated form of histone 3 can be used in combination with a fluorescently labeled secondary antibody. The percentage of mitotic cells is then identified by flow cytometry.

1. Transfer 3 – 5 million cells in a 15 mL conical tube (see Note 8).
2. Spin the cells for 5 minutes at 1000rpm at 4°C. Remove the medium.
3. To fix the cells resuspend the cell pellet in 1 mL of 3,7 % formaldehyde in PBS and incubate at room temperature for 15 minutes.
4. Spin the cells for 5 minutes at 1000rpm at 4°C.
5. Remove the fixative and resuspend the cells in 250 µL of PBS.
6. Pre-chill tubes on ice for approximately 2 minutes.
7. Permeabilise the cells by slowly adding 2,25 ml of ice cold methanol and vortexing at the same time.
8. Incubate on ice for 30 minutes (see Note 9).
9. Spin the cells for 5 minutes at 1000rpm at 4°C.
10. Resuspend in 1 ml of Incubation Buffer and allow the cells to rehydrate for 5 minutes at room temperature.
11. Spin the cells for 5 minutes at 1000rpm at 4°C.
12. Repeat step 10, but incubate the cells in the Incubation Buffer for 20 minutes.
13. Spin the cells for 5 minutes at 1000rpm at 4°C.
14. Incubate the cells with primary antibody against phosphorylated histone H3 overnight at 4°C (see Note 3). Dilute the antibody (1:250) in the Incubation Buffer and resuspend the cell pellet in 50 – 60 µL of antibody solution.
15. Wash the cells twice with 1 mL of Incubation Buffer and spin the cells down after each wash (5min, 1000rpm, 4°C)
16. Incubate the cells in the dark in 50 µL of fluorophore conjugated secondary antibody diluted in the Incubation Buffer.
17. Wash the cells twice with 1 ml of Incubation Buffer and spin the cells down after each wash (5min, 1000rpm, 4°C).
18. Resuspend the cell pellet in 500 µL of PBS and store the cell suspension a 4°C in the dark until flow cytometry analysis.
19. Analyze the samples on flow cytometer. Using Cell Quest or Flow Jo software determine the percentage of cells within the analyzed population which stained positively for mitotic marker (green channel) (Figure 3).

Immunolabeling of mitotic cells - fluorescent microscopy

The mitotic cells after exposure to DNA damaging insults can also be observed and counted manually under a fluorescent microscope. For this purpose we include the protocol for immunolabelling of cells fixed on microscope slides.

1. Insert poly L-lysine coated microscope cover slips into the wells of a 6-well plate or 35mm dishes.
2. Seed the cells on coated cover slips and expose to DNA damaging agents as described in protocol 3.3.
3. Wash the wells twice with PBS.
4. Add 1mL of formaldehyde solution to each well. Incubate for 15 minutes at room temperature to fix the cells.
5. Remove the fixative and wash the wells 3 times for 5 minutes with PBS.
6. Add 1mL of 0,2 % Triton-X100 to each well. Incubate for 15 minutes at room temperature to pereabilize the cells.
7. Incubate the coverslips with 3 % BSA solution for 1 hour.
8. Dilute the primary antibody against phosphorylated histone H3 1:250 in 3 % BSA. Apply a droplet (25 - 40 μL) of antibody solution on each coverslip and cover with a piece of parafilm.
9. Incubate overnight at 4°C in a humid chamber.
10. Remove parafilm pieces and wash the wells 3 times 5 minutes with PBST.
11. Dilute the Alexa Fluor 488 conjugated secondary antibody (1:1000) in 3 % BSA. Apply a droplet (25 - 40 μL) of antibody solution on each coverslip and cover with a piece of parafilm.
12. Incubate 1 - 2 hours at room temperature.
13. Remove parafilm pieces and wash the wells 3 times 5 minutes with PBST.
14. Apply a droplet (25 - 40 μL) of DAPI solution on each coverslip and cover with a piece of parafilm. Incubate for 15 minutes to satin DNA in the cells.
15. Remove parafilm pieces and wash the wells 3 times with PBS.
16. Gently lift the cover slips from the wells and mount on microscope slides with a droplet of Citifluor mounting solution. Apply slight pressure on the cover slips to remove the excess of mounting medium. Cover the edges of each cover slip with a layer of nail polish.
17. Analyze the slides using a fluorescent microscope. To calculate the percentage of cells in mitosis, at least 1000 cells should be counted for each condition.

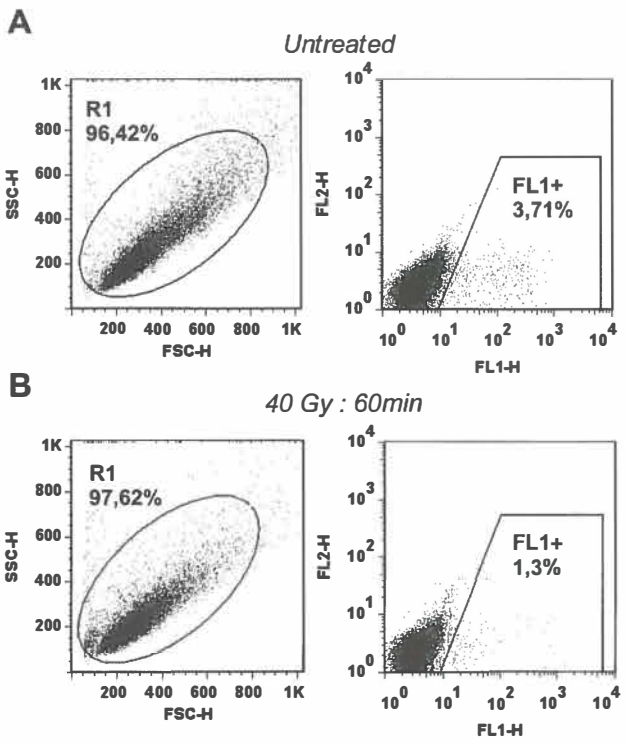

Figure 3. Flow cytometry analysis of *Drosophila* S2 cells stained for mitosis specific phosphorylated histone H3.

S2 cells were fixed and stained with anti phosphorylated histone H3 antibody in combination with Alexa 488 conjugated secondary antibody. (A and B) Left images: Forward Scatter/Side Scatter plot shows homogeneous distribution of cells gated within one population (marked by R1; 98.51% of all cells). Right images: FL1/FL2 plot shows the fluorescence signal of all cells selected in R1. The boxed area indicates a distinct population of mitotic cells, which stained positively and give a signal in FL1 (FITC signal) channel (marked FL1+). a shows the amount of mitotic cells under control culturing conditions (3.71% of all cells show a FITC signal above threshold and therefore are considered as cells in mitosis). b shows the amount of mitotic cells after ionizing radiation (40 Gy, and 60 min recovery), (1.3% of all cells show a FITC signal above threshold and therefore are considered as cells in mitosis).

DNA staining – determination of the cell cycle distribution

1. Transfer 1 – 2 million cells in a conical tube.
2. Wash cell pellet twice with 1 mL of PBS by spinning down the cells after each wash (5min, 1000rpm, 4°C).
3. Fix the cells in 2 mL of 80% ethanol:aceton (1:1). Add the fixative drop-wise vortexing the tube at the same time.
4. Incubate overnight at 4°C.
5. Spin the cells for 5 minutes at 1000rpm at 4°C.
6. Wash the cell pellet once with 5 mL PBS.
7. Resuspend the cell pellet in 20 µL of DNase free RNase A and incubate for 30 minutes in 37°C.
8. Add 400 µL of Propidium Iodide solution.
9. Incubate at room temperature for 1 hour or overnight at 4°C.
10. Analyze samples by flow cytometry in the presence of the dye.
11. Using FlowJo or ModFit software determine the distribution of cells in G1, S and G2/M phase.

Notes

1. Serum purchased from different suppliers may differ greatly. It is recommended to test each new batch of serum for possible cell toxicity. Serum should be heat inactivated in 65°C for 30 minutes and stored in aliquots at – 20°C. Repeated freezing and thawing should be avoided.
2. We have tried a number of serum free media designed for serum free insect cell cultures for our RNAi experiments. Optionally to classical Schneider's *Drosophila* Medium with L-Glutamin, Express Five SFM Medium (Invitrogen) can also be used with a similar transfection efficiency. Add 9mL of 200 mM L-Glutamine to each 100mL of Express Five SFM Medium prior to use.
3. A wide selection of primary antibodies against phosphorylated histone H3 as well as fluorophore conjugated secondary antibodies is commercially available. Alexa 488 (green) may as well be replaced with other fluorophore if a change of the color is required.
4. Choosing an appropriate gene fragment for a design of dsRNA is crucial for a good RNAi experiment. Online software tools, such as E-RNAi (http://e-rnai.dkfz.de/) can be very helpful by designing such a template [24]. Special attention should be paid to avoiding off-target effects. If possible a dsRNA should not contain any 19-mer homology to a gene other than the gene of interest. Two non-

overlaping sequences acting on distant places of the target mRNA can also be used.

5. In case of a low yield multiple PCR reactions should be combined, purified and eluted with one volume.
6. The optimal dsRNA concentration should always be determined experimentally, however the given ranges should, in most cases, result in an efficient knockdown. We recommend to use 10 - 40 nM dsRNA in 1 mL of serum free medium (see Table 1). The lowest effective concentration should always be used to avoid non specific gene targeting.
7. A successful knock-down is usually observed after 3 days of RNAi. However, this time may differ greatly depending on the stability and half-life of the target protein. We routinely perform very efficient RNAi experiments in which more than 90 % knockdown can be observed during a period of 3 - 9 days of RNAi treatment.
8. This number of cells is more than enough for the flow cytometry analysis. We do not recommend decreasing this number since after centrifugation less cells will produce only a small pellet which can be easily lost (especially by inexperienced hands) during the 2-day staining protocol.
9. Cells in 90 % methanol can be stored for at least a couple of weeks in -20°C.

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Impaired cell cycle progression in response to decreased Coenzyme A levels in *Drosophila* S2 cells

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Manuscript in preparation

Introduction

Coenzyme A (CoA) is a central and key player in cellular metabolism. As a carrier of acyl groups, CoA constitutes an essential cofactor necessary for approximately 4 % of all reactions of intermediary metabolism (reviewed in [1]). CoA is synthesized *de novo* from vitamin B5 (pantothenic acid) in a linear pathway, which is highly conserved between species. The pathway consists of the subsequent actions of 5 enzymes and pantothenate kinase is involved in the first conversion step of CoA biosynthesis [1] (Figure 1A). Although the biochemical function of CoA has been studied for the past 60 years, surprisingly little is known about the consequences of altered CoA biosynthesis in higher eukaryotes. The importance of this knowledge became recently evident because mutations in human pantothenate kinase 2 lead to the severe neurodegenerative disorder PKAN (pantothenate kinase-associated neurodegeneration) [2]. PKAN is a devastating disease with a complex an largely unresolved pathophysiology [3].

To proliferate a cell must constantly cycle between interphase and mitosis, carrying out and synchronizing a number of complex processes (such as replication of DNA, synthesis of organelle and membrane components). Thus, precise fine-tuning between the metabolic status and the cell cycle machinery is essential. Considering the central place of CoA on the crossroads of many metabolic pathways, it is highly probable that impairment of CoA metabolism affects regulation of cell proliferation and survival. However, whether and how CoA levels influence cell cycle regulation is currently unknown.

Here we aim to investigate the effect of impaired CoA metabolism on cell proliferation and cell cycle progression of *Drosophila melanogaster* cultured cells. We use an RNAi approach to down-regulate the *Drosophila* pantothenate kinase dPANK/Fbl in order to interfere with the CoA *de novo* biosynthesis pathway. Our results show that impaired CoA biosynthesis strongly reduces cell proliferation and cell cycle distribution profiles are changed in dPANK/Fbl-depleted cells. The cells accumulate in the G2 phase of the cell cycle and entrance into mitosis is delayed. This delayed progression of dPANK/Fbl-depleted cells through G2 and M phases coincides with decreased levels of mitotic cyclines. Thus, our results show a strong influence of impaired CoA metabolism on various specific cell cycle events.

Results

dPANK/Fbl down-regulation affects cell proliferation

To investigate the effect of impaired CoA *de novo* biosynthesis on the proliferation and survival in *Drosophila* we used Schneider's S2 cells for which highly efficient RNAi protocols have been established [4] and in which conserved cell cycle regulation is present [5-8]. These macrophage-derived cells are further characterized by a fast proliferation rate and the ability to reach high densities in culture, which enables biochemical studies that require large quantities of highly similar cells. Using double stranded RNA we down-regulated dPANK/Fbl protein to below detection levels (Figure 1B). The protein was undetectable between day 4 and 9 after the dsRNA treatment, allowing us to investigate relatively long-term effects of dPANK/Fbl depletion. Under these circumstances we investigated the proliferation rate of control and RNAi-treated cells. dPANK/Fbl-deficient cells increased in cell number at much slower rates compared to untreated cultures (Figure 1C). To investigate whether the decrease in cell numbers was due to higher incidents of cell death, we measured cell viability using a Trypan Blue exclusion assay, however no significant increase in the percentage of dead cells was observed in dPANK/Fbl RNAi-treated cells. Furthermore, we were unable to significantly detect a dPANK/Fbl RNAi-induced increase in the sub-G1 population in the flow cytometry profiles (data not shown), therefore there is no indication that dPANK/Fbl-depleted cells undergo increased apoptosis.

Together these data demonstrate that dPANK/Fbl down-regulation in S2 cells affects cell numbers most probably as a result of a reduced cell proliferation rate rather than increased cell death or apoptosis.

dPANK/Fbl depletion increases the proportion of cells in G2 phase and delays progression through G2 and M phase

To further investigate the decrease in cell proliferation, we used flow cytometry to investigate cell cycle profiles of dPANK/Fbl RNAi-treated cells. We found that, in comparison to control cultures, after dPANK/Fbl depletion an increased population of the cells accumulated in G2/M (Figure 2). 29 % of control cells were in G2/M phase, whereas 45 % of dPANK/Fbl RNAi-treated cells were in G2/M. Under these conditions of dPANK/Fbl depletion, the percentage of cells in S phase reduced from 19,6 % to 13,7 % and the proportion of cells in G1 phase decreased from 49,7 % to 40,6 %.

To further analyze cell cycle profiling of dPANK/Fbl-depleted cells we used BrdU staining to specifically identify cells in S phase and subsequently track their progression through the cell cycle (Figure 3). Pulse labeled BrdU positive cells under control conditions passed from S phase to G2 within 3 hours after the BrdU pulse, followed by progression through mitosis and back into G1 and S phase (Figure 3, left panel). BrdU

positive dPANK/Fbl-depleted cells labeled under the same conditions reached the G2 phase at the same speed (3 hours), however delayed progression through G2/M into G1 was observed (Figure 3, right panel). Indicating that dPANK/Fbl depleted cells progress normally from S-phase to G2 phase, but further accumulate in G2/M and a 6 hour delay is observed under conditions of dPANK/Fbl depletion compared to control cells before the BrdU positive population reaches G1.

dPANK/Fbl was initially discovered in a male sterility screen as a novel gene required for normal mitotic and meiotic divisions *in vivo* [9]. Afshar et al. reported that in neuroblasts (neuronal progenitor cells) from developing homozygous *dPANK/fbl* null larval brains around 80 % fewer mitotic figures were observed compared to wild type larval brains. These data indicate that also in *dPANK/fbl* mutants abnormal cell cycle events occur.

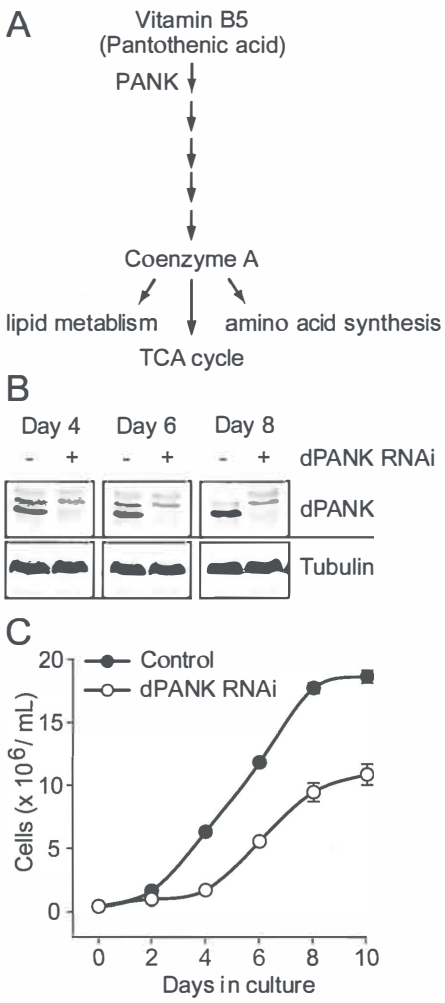

Figure 1. Impaired CoA biosynthesis in *Drosophila* S2 cells slows cell proliferation.

A, A simplified scheme of the *de novo* biosynthesis of Coenzyme A. Vitamin B5 (pantothenic acid) is phosphorylated by pantothenate kinase (PANK) in the first and rate-limiting step of the pathway. CoA is synthesized after four additional steps. Functioning as a carrier of acyl groups, CoA is essential in many metabolic pathways, including the Creb's cycle, lipid metabolism and synthesis of several amino acids. B, dPANK/Fbl protein was down-regulated in S2 cells using dsRNA. Efficiency of the knock-down was monitored 4, 6 and 8 days after dsRNA treatment using a specific dPANK/Fbl antibody. Tubulin was used as a loading control. C, Proliferation of control and dPANK/Fbl-depleted cells was followed for a period of 10 days. dPANK/Fbl RNAi-treated cells increased in cell numbers at slower rate than control cells. Data points represent mean ± S.E.M.

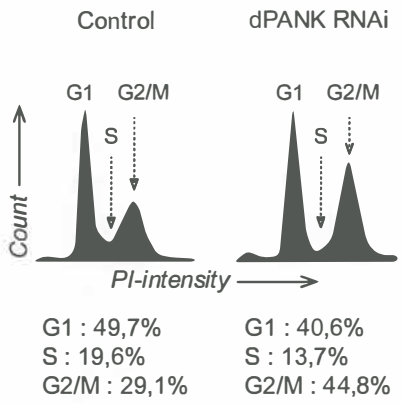

Figure 2. dPANK/Fbl RNAi-treated cells accumulate in G2 phase of the cell cycle.

Flow cytometer cell cycle profiles of control and dPANK/Fbl RNAi-treated cells were obtained after propidium iodide staining. Percentage of cells in G1, S and G2/M phases was determined and indicated below the plots. Data shown are representative of 3 independent experiments.

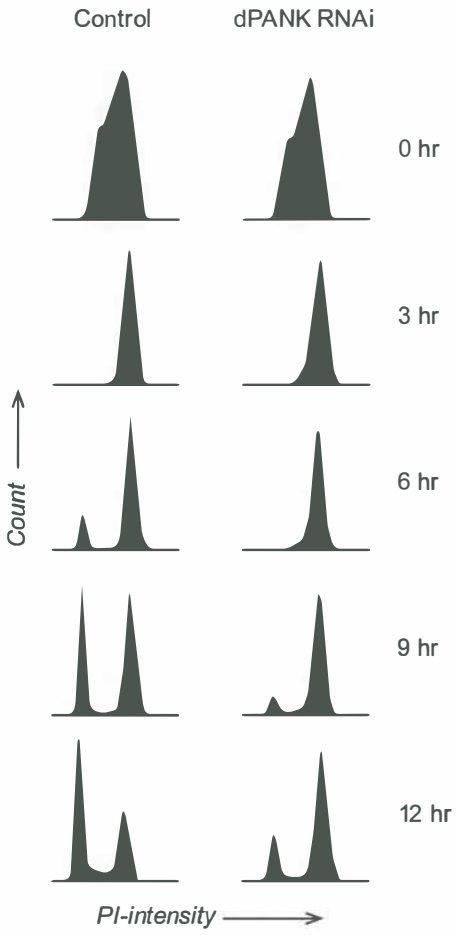

Figure 3. BrdU pulse-labeled cells progress slower through G2/M under conditions of impaired CoA biosynthesis.

Control and dPANK/Fbl RNAi cells were pulse-labeled with BrdU for 15 minutes and then shifted to BrdU-free medium for an indicated period of time. Cell cycle profiles of BrdU positive cells were recorded by flow cytometry. Slowed progression of dPANK/Fbl RNAi-treated cells through G2/M phase of the cell cycle was apparent after 6, 9 and 12 hours from the BrdU pulse.

Expression of mitotic cyclins and entry into mitosis are affected upon dPANK/Fbl down-regulation

Our data showing a delayed progression through G2/M suggest that entrance into mitosis is delayed or mitosis itself is prolonged. To test this further we used flow cytometry to analyze the mitotic index of control and dPANK/Fbl RNAi-treated S2 cells (Figure 4). In exponentially growing control cultures around 5 % of cells stained positively for phosphorylated histone 3 (a mitosis-specific marker, [10]). However, dPANK/Fbl depletion resulted in a 50 % decrease in the mitotic index. Additionally, we treated cells with colchicine which arrests cells in mitosis [11] (Figure 4). After an 8-hour-treatment with colchicine a 4,5 fold increase in the mitotic index could be observed in control cells. This increase reflects the number of cells that have entered mitosis within 8 hours. In contrast, the mitotic index of dPANK/Fbl-depleted cells treated with colchicine increased less than 2 fold. In summery these results indicate that one of the consequences of the impaired CoA biosynthesis in actively cycling cells is a delayed entry into mitosis and these data are consistent with the studies performed in *dPANK/fbl* mutants [9].

Dynamic regulation of the cell cycle machinery ensures that progression from one cell cycle phase to the other is properly timed. The process is tightly controlled by master regulatory proteins, such as cyclins and cyclin dependent kinases (CDKs). CDKs are constitutively expressed and regulated by phosphorylation, whereas the activity of cyclins is primarily determined by orchestrated changes in their expression levels [12]. Thus, having observed the changes in cell cycle distribution in dPANK/Fbl-depleted *Drosophila* S2 cells, we sought to investigate the expression dynamics of various cyclins in these cells. In order to obtain an efficient amount of cells for a biochemical analysis, we used a double thymidine block to synchronize and arrest cells in S phase [11]. Synchronized cells were released into normal growth media and expression levels of cyclin A, cyclin B and phospho-H3 were determined at different time-points. Both cyclin A as well as cyclin B accumulate during G2 phase with a maximum expression peak at the initiation of mitosis. Further their sequential degradation contributes to the progression through mitosis such that the degradation of cyclin A is completed earlier in mitosis than the degradation of cyclin B [13-15]. These normal dynamics of cyclin A and cyclin B expression were indeed observed in control S2 cells after the double thymidine block release (Figure 5). In addition, the normal progression of control cells through mitosis was evident by increased levels of phospho-H3 (p-H3). Cyclin A levels first increased and finally decreased sharply at the beginning of mitosis, followed by the degradation of cyclin B at later time-points. However, under these conditions dPANK/Fbl-depleted cells did not accumulate cyclin A, neither cyclin B. Initial levels of both cyclines were around 30 – 40 % decreased as compared to control cells. A small drop in cyclin A expression was observed in dPANK/Fbl-depleted cells at the time points comparable to cyclin A degradation in control cells. However, cyclin B levels in dPANK/Fbl-deficient

cells remained low and constant throughout the experiment. These cells did not accumulate phospho-H3 either, further underscoring that impaired CoA biosynthesis hampers timely entrance into mitosis.

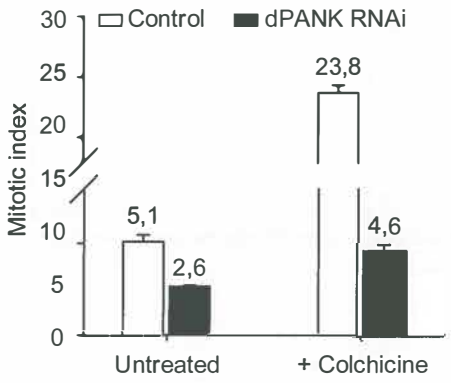

Figure 4. Mitotic index of dPANK/FbI-deficient cells is decreased as compared to control cultures.

Exponentially growing control and dPANK/FbI RNAi-treated cells were left untreated or were treated with 20 µg/mL colchicine for 8 hours. Mitotic cells (marked by a positive phospho-histone 3 staining) were analyzed by flow cytometry and the mitotic index was determined as a percentage of cells in mitosis within the total population. Data points represent mean (indicated above bars) ± S.E.M.

Discussion

Our data demonstrate that impaired CoA biosynthesis affects cell proliferation and cell cycle regulation. The progression from one cell cycle phase to the next is controlled by complex signaling pathways which ensure that the complete cycle is properly timed and fine-tuned with the metabolic state/needs of a cell (for an extensive review on the metabolic control of cell cycle progression see [16]). Here we show that in *Drosophila* cultured cells down-regulation of pantothenate kinase, a rate limiting enzyme in the *de novo* biosynthesis of CoA, impairs cell proliferation and slows down the progression of cells though G2 and M phase.

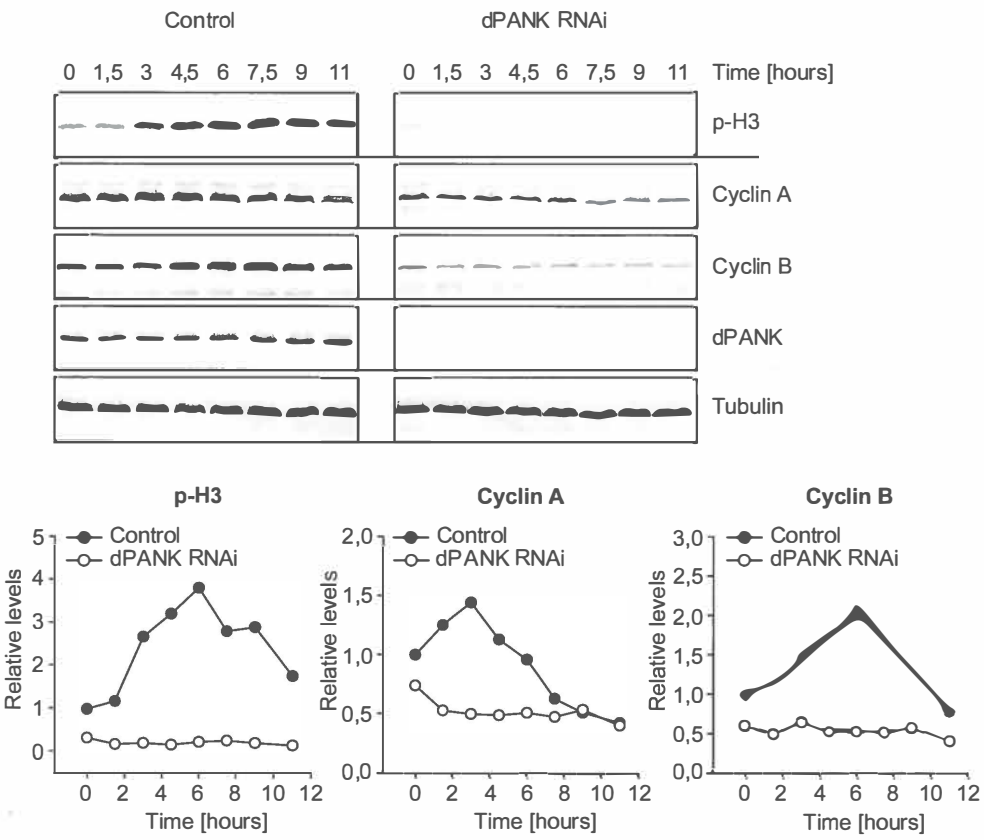

Figure 5. Cyclin A and cyclin B expression is dysregulated in a dPANK/Fbl-deficient background.

Control cells and cells treated with dPANK/Fbl dsRNA were synchronized by a double thymidine block. Samples were collected at indicated times after the release from the thymidine block. Phosopho-histone 3 (p-H3), cyclin A and cyclin B levels were determined by Western blot analysis using specific antibodies. Tubulin was used as a loading control and for the quantification of the relative levels of proteins. Values for control cells at the 0 hour time point were set as 1. Blots and graphs are representative of two independent experiments.

The decrease in proliferation rate observed in dPANK/Fbl-depleted cells is in agreement with previously published data. Analogous growth delay as a result of pantothenate kinase deficiency has been reported in bacteria [17, 18]. Studies on pantothenate kinase deficient bacterial strains indicated that the onset of growth stasis in CoA-depleted cells is mostly due to their inability to synthesize amino acids and proteins and not (or to a smaller extend) due to abnormalities in lipid metabolism [17]. If a similar mecha-

nism of growth delay applies to *Drosophila* cells, this could explain our observation that the expression of cell cycle regulator proteins (cyclin A and cyclin B) in dPANK/Fbl-deficient cells is decreased.

Impaired protein as well as lipid metabolism have already been linked to cell cycle defects [16]. Thus, considering the wide range of metabolic processes in which CoA is involved, further studies are necessary to reveal precise mechanisms and molecular pathways behind the cell cycle abnormalities induced by impaired pantothenate kinase activity. Interestingly, cell cycle regulation has also recently been linked to acetyl-CoA metabolism via histone acetylation [19]. Wellen et al show that the ATP-citrate lyase, an enzyme converting citrate and CoAinto acetyl-CoA, is necessary to induce histone acetylation during a re-entry into the cell cycle following serum starvation. Thus, it would be interesting to investigate if in *Drosophila* S2 cells histone acetylation is regulated in a cell cycle-dependent manner, similarly to other cell lines [20]. If so, it is likely that impaired CoA synthesis would impair cell cycle progression due to reduced availability of acetyl-CoA for histone acetylation (for details on histone acetylation in CoA-deficient background, see chapter 5 of this thesis).

It is likely, however it remains to be tested, that the cell cycle abnormalities presented here may have physiological significance on the whole-organism level. Mitotic abnormalities can be observed in *Drosophila* null *dPANK/fbl* mutants as well as in hypomorph mutants (with decreased dPANK/Fbl expression) [9, 21], however these were only observed during development, spematogenesis and oogenesis. This can be explained because during these processes numerous cell divisions take place. Mutations in human PANK2 result in a severe neurodegeneration, however no developmental defects in PKAN patients were reported, since the disease onset is after birth (during childhood or adulthood) [22]. Nonetheless, it cannot be excluded that PANK2 impairment affects actively cycling cells (stem cells) in an adult organism, which could influence negatively the plasticity and regenerative capacity of tissues.

Materials And Methods

Cell culture - *Drosophila* Schneider's S2 cells (Invitrogen) were cultured at 25°C in Schneider's medium (Invtrogen) supplemented with 10 % heat-inactivated fetal calf serum (Gibco) and penicillin/streptomycin (Invitrogen). Cells in the exponential phase of growth were used for the experiments.

dPANK/Fbl RNAi - DNA template (a fragment of for *dPANK/fbl* gene) for dsRNA synthesis was PCR amplified using primers (fwd – CGTGATACGCACCTACAGATG, rv – GCCATTGGACCAGAACTCCAT) containing 5'T7 RNA polymerase binding site (TAATACGACTCACTATAGGG). dsRNA was obtained by *in vitro* transcription and purified using MegaScript RNAi Kit (Ambion). 2 pmols of DNA templates were used per 20 uL *in vitro* transcription reaction. dsRNA treatment was carried out as described previously [4]. Cells were incubated in serum-free medium containing 40nM dsRNA for 1 hour, following by addition of serum containing medium. After 4 days, cells were subcultured to a density of 0,4 million / mL and maintained for additional 3-4 days. Various assays were performed at day 7 or 8 after the initial treatment with dsRNA.

Cell cycle analysis - For cell cycle profiling cells (2 million / sample) were centrifuged, washed once with PBS and fixed in 80% ethanol:aceton. Fixed cells were stored at 4°C for at least 12 hours, then washed twice with PBS and incubated for 30 minutes at 37°C in 25 μL of 10 mg/mL DNase-free RNase A (Sigma). 25 ug/mL propidium iodide (Sigma) in PBS was added (400 μL) and cells were stained at room temperature for 30 minutes (in the dark). DNA content was analyzed on FACScalibur flow cytometer using Cell Quest (Becton Dickinson).

BrdU pulse-chase experiments – Control or RNAi treated cells were cultured until day 7 as described above. On day 7 cells were subcultured into a density of 0,8 million/mL. 10-12 hours later 15 μM BrdU (Sigma) was added for 15 minutes and then the cells were resuspended and washed two times with PBS by centrifugation. Cells were resuspended in BrdU-free medium to a density of 1,3 million/mL and plated in six well plates. Cells were collected at specified time-points, washed with PBS, fixed in ice-cold 70 % ethanol and stored at 4°C until all time-points were collected. Fixed cells were washed twice in PBS, permeabilised for 30 minutes in 2 M HCl and then washed once in PBS and twice in PBS-BT (PBS + 0,5 % BSA + 0,2 % Tween 20). Cells were incubated with mouse anti-BrdU (Sigma, 1:500) overnight at 4°C and then washed twice with PBS-BT and incubated for 1 hour at room temperature with Alex-488 conjugated goat anti-rabbit secondary antibodies (Invitrogen, 1:1000 dilution). Finally, cells were washed with PBS, treated with RNase A and stained with propidium iodide as described above.

Immunolabeling of mitotic cells - To determine the mitotic index 3-5 million cells/sample were centrifuged, washed once with PBS and fixed in 3,7 % formaldehyde in PBS for 15 minutes at room temperature. Fixed cells were washed with PBS and centrifuged, cell pellets were resuspended in 250 µL of pre-chilled PBS and 2,25 mL of ice-cold methanol was added while vortexing to permeabilise the cells. After 30 min incubation on ice, cells were washed 2 times in PBS-BT (PBS + 0,5 % BSA + 0,2 % Tween 20) followed by overnight incubation with a rabbit anti-phospho-histone 3 antibody (Cell Signaling, 1:500 dilution). Finally cells were washed two times with the incubation buffer and incubated for 1 hour at room temperature with Alex-488 conjugated goat anti-rabbit secondary antibodies (Invitrogen, 1:1000 dilution). After washing one time with PBS cells were analyzed on FACScalibur flow cytometer using Cell Quest (Becton Dickinson).

Double thymidine block experiments – Cells were synchronized by double block with 2 mM Thymidine (Sigma) according to the following scheme: 10 hr block - 6 hr release - 8 hr block. After the second block, cells were washed twice with PBS, resuspended in thymidine-free medium to the density of 1,3 million/mL and plated in six well plates. Cells were collected at specified time-points and processed for Western blot analysis.

Western blot analysis - Equal numbers of cells were centrifuged, washed once with PBS and lysed immediately in 1 X Laemmli Sample Buffer (62,5 mM Tris/HCl pH 6,8; 2 % SDS; 10 % glycerol; bromophenol blue). Samples were further sonicated and boiled for 4 min with 5 % 2-mercaptoethanol. Protein extracts were run on 12,5 % SDS-Page gels, transferred onto nitrocellulose membranes and blocked with 5 % milk in PBS/0,1 % Tween, following by an overnight incubation with primary antibodies. The following antibodies were used: rabbit anti-dPANK/Fbl [21], mouse anti-tubulin (Sigma), mouse anti-cyclin A (A12, Developmental Studies Hybridoma Bank), mouse anti-cyclin B (F2F4, Developmental Studies Hybridoma Bank), rabbit anti-phospho-histone 3 (Ser 10) (Cell Signaling). HRP-conjugated secondary antibodies (Amersham) were used and detection was performed using enhanced chemiluminescence. Band intensities were quantified with Adobe Photoshop.

Acknowledgements

We thank Geert Mesander and Henk Moes for technical assistance during the flow cytometry measurements.

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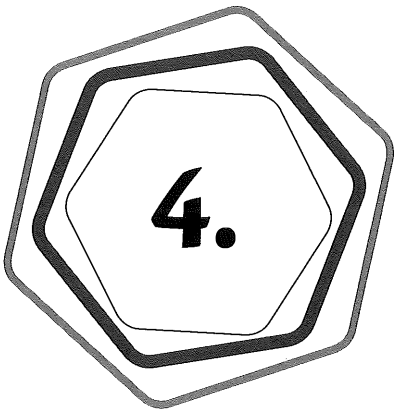

Pantethine rescues a *Drosophila* model for pantothenate kinase-associated neurodegeneration

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Proc Natl Acad Sci U S A. 2010 Apr 13;107(15):6988-93

Abstract

Pantothenate kinase–associated neurodegeneration (PKAN), a progressive neurodegenerative disorder, is associated with impairment of pantothenate kinase function. Pantothenate kinase is the first enzyme required for *de novo* synthesis of CoA, an essential metabolic cofactor. The pathophysiology of PKAN is not understood, and there is no cure to halt or reverse the symptoms of this devastating disease. Recently, we and others presented a PKAN *Drosophila* model, and we demonstrated that impaired function of pantothenate kinase induces a neurodegenerative phenotype and a reduced lifespan. We have explored this *Drosophila* model further and have demonstrated that impairment of pantothenate kinase is associated with decreased levels of CoA, mitochondrial dysfunction, and increased protein oxidation. Furthermore, we searched for compounds that can rescue pertinent phenotypes of the *Drosophila* PKAN model and identified pantethine. Pantethine feeding restores CoA levels, improves mitochondrial function, rescues brain degeneration, enhances locomotor abilities, and increases lifespan. We show evidence for the presence of a *de novo* CoA biosynthesis pathway in which pantethine is used as a precursor compound. Importantly, this pathway is effective in the presence of disrupted pantothenate kinase function. Our data suggest that pantethine may serve as a starting point to develop a possible treatment for PKAN.

Introduction

CoA is a ubiquitous and essential cofactor for various metabolic reactions, including the tricarboxylic acid cycle and fatty acid metabolism [1]. The canonical pathway leading to *de novo* synthesis of CoA starting at vitamin B5 (also known as pantothenate or pantothenic acid, further referred to as VitB5) is well known. All genes encoding the CoA biosynthetic enzymes have been identified and are highly conserved between different species [2-6] (Figure 1A).

The biosynthesis of CoA, especially the CoA biosynthetic enzyme pantothenate kinase (PANK; EC 2.7.1.33), received renewed interest after the discovery that the Hallervorden-Spatz syndrome, a hereditary disease mainly affecting children, is caused by a mutation in the human PANK2 gene, one of the four human pantothenate kinase genes (PANK1-4), rendering the enzyme inactive [7]. Accordingly, this syndrome has been referred to pantothenate kinase–associated neurodegeneration (PKAN). This finding uncovered a completely unknown role of CoA biosynthesis in cellular functioning. Patients with the autosomal recessive disorder PKAN show progressive impairment of speech, locomotor, and cognitive function [8]. The pathophysiology of PKAN is not understood, and there is no cure to revert or delay the neurodegeneration. It is not known whether there are decreased levels of CoA in the affected tissues and thus whether decreased levels of CoA coincide with impaired neurological and locomotor function. Although a Pank2 mouse knock-out has been generated, this murine model did not show any signs of neurodegeneration [9], leaving the question unanswered as to whether decreased levels of CoA are causative in PKAN.

Recently, we and others have demonstrated that mutations in *Drosophila* CoA biosynthesis enzymes, including the *Drosophila* homolog of PANK2 (further referred to as *dPANK/fbl* mutants), induce a neurodegenerative phenotype; and these flies can be used as a model for PKAN-related research [2, 4, 10]. *Drosophila* is not only a powerful model to understand the mechanisms of various human neurodegenerative diseases [11], but *Drosophila* disease models are also of value to identify compounds that are able to rescue disease-associated characteristics [12].

In the present study, we used the *Drosophila dPANK/fbl* mutants and dPANK/Fbl down-regulated *Drosophila* cultured S2 cells to address the following questions: (i) Does depletion of dPANK/Fbl correlate with decreased levels of CoA? (ii) If dPANK/Fbl depletion does induce decreased levels of CoA, are there ways to restore CoA levels in this background? (iii) If we are able to restore CoA levels, does this lead to a rescue of the phenotypes induced by dPANK/Fbl depletion?

Our results show that dPANK/Fbl depletion results in a significant decrease of CoA. Furthermore, we tested several compounds for their potential to restore CoA levels in the presence of impaired dPANK/Fbl function. One of the compounds tested was pantethine (the disulphide of pantetheine). Previously, it has been demonstrated that purified enzymatic extracts were able to convert both pantethine and pantetheine into 4'-phosphopantetheine [13-15], an intermediate in the canonical *de novo* CoA biosynthesis pathway (Figure 1A). However, it has never been tested whether this alternative pathway is functional in a PANK-impaired background, although this knowledge is highly relevant in light of a possible PKAN therapy. Feeding pantethine to *dPANK/fbl* mutant flies or adding pantethine to dPANK/Fbl–down-regulated S2 cells restored CoA levels and rescued nearly all tested phenotypes, including the neurodegenerative phenotype. Our data further indicate that pantethine rescued mitochondrial abnormalities in hPANK2-depleted mammalian cells. Our results strongly suggest that pantethine can serve as a precursor compound to generate CoA even in the absence of a functional pantothenate kinase. Our findings may serve as a starting point to develop a possible treatment for PKAN.

Results

dPANK/fbl mutant flies show reduced levels of CoA

Pantothenate kinase is the enzyme required for the first step in the canonical biosynthetic route of CoA (Figure 1A). In hypomorphic *dPANK/fbl* mutant flies the dPANK/Fbl protein content was severely decreased (Figure 1C). Although there is a dynamic turnover of CoA in numerous intracellular metabolic reactions, there is only one route known that leads to the *de novo* synthesis of CoA [1]. Therefore, we hypothesized that low levels of CoA caused the phenotype of *dPANK/fbl* mutants. Indeed, HPLC analysis clearly revealed significantly lower levels of CoA in homozygous *dPANK/fbl* mutants compared with wild type (Figure 1B). To further test the effect of impaired function of *dPANK/fbl* on CoA levels, dPANK/Fbl protein levels were down-regulated in *Drosophila* S2 cells by RNAi (experimental setup in Fig. S2). Four days after the addition of *dPANK/fbl* dsRNA, dPANK/Fbl protein levels were strongly decreased (Figure 1D). Under these circumstances, CoA levels were also significantly decreased to 24% of levels of control cells (Figure 1E), and cell counts were significantly lower as compared with control cells (Figure 2A). This suggested that *de novo* synthesis of CoA is required for maintenance of normal levels of CoA in *Drosophila* cells and accordingly for normal cell growth in culture.

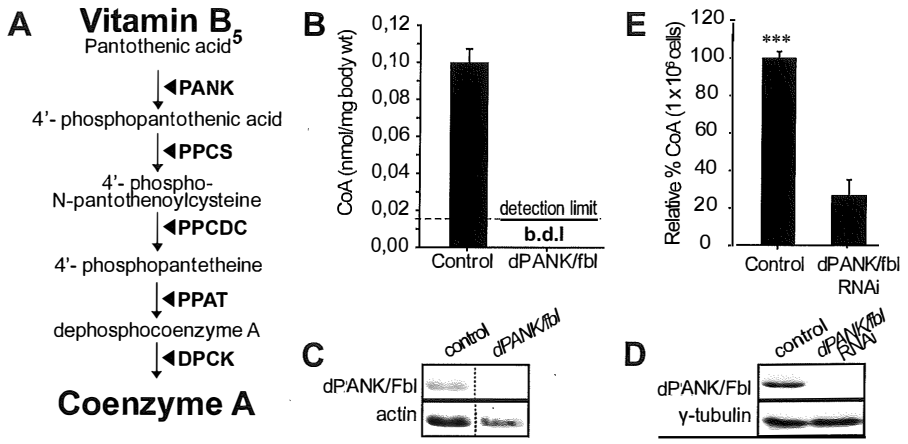


Figure 1. dPANK/Fbl impairment leads to reduced levels of CoA.

A, Scheme of canonical *de novo* CoA biosynthesis pathway. Vitamin B5 (pantothenic acid) is converted into CoA by the consecutive action of five enzymes: PANK, pantothenate kinase (EC2.7.1.33); PPCS, phosphopantotenoylcysteine synthase (EC6.3.2.5); PPCDC, phospho-N-pantothenoylcysteine decarboxylase (EC4.1.1.36); PPAT, phosphopantetheine adenylyltransferase (EC2.7.7.3); and DPCK, dephospho-CoA kinase (EC2.7.1.24)). B, HPLC was used to measure levels of CoA in wild-type adult flies and in *dPANK/fbl* homozygous mutants at 6 days of age. C, Western blot analysis was used to examine levels of dPANK/Fbl protein in wild types and *dPANK/fbl* mutants at 6 days of age. Actin was used as a loading control. D, Western blot analysis was used to measure dPANK/Fbl protein levels in S2 cells 4 days after addition of *dPANK/fbl* dsRNA. As a control, cells were treated with mock dsRNA. (E) HPLC was used to detect levels of CoA in control *Drosophila* Schneider's S2 cells and in S2 cells 7 days after *dPANK/fbl* RNAi treatment. ***P < 0.001 (Student's t test).

Pantethine addition restores normal growth of dPANK/Fbl-depleted cells

Our data strongly suggested that reduced levels of CoA might be the primary cause for the decreased cell count of dPANK/Fbl-depleted cells and for the mutant phenotype of *dPANK/fbl* homozygous flies. Accordingly, restoring CoA levels in dPANK/Fbl-depleted cells and in *dPANK/fbl* mutants should lead to a rescue of the related phenotypes. We checked several compounds related to CoA biosynthesis (CoA, VitB5, and pantethine) for their ability to rescue growth of dPANK/Fbl-depleted S2 cells and, when successful, for their ability to restore CoA levels. CoA was tested because adding CoA as a supplement may directly restore CoA levels. VitB5 was tested because adding large doses of VitB5 may compensate for decreased activity of dPANK/Fbl enzyme in

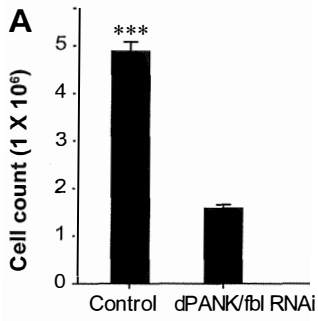
a *dPANK/fbl* hypomorphic mutant background. Pantethine was tested because previously it has been reported that pantethine can be converted into the normally occurring CoA intermediate 4'-phosphopantetheine [13-15] (Figure 1A). Our results showed that although high concentrations of all compounds were toxic, CoA and pantethine were effective in restoring cell counts of dPANK/Fbl-depleted cells in a concentration-dependent manner, whereas VitB5 was ineffective (Figure 2 B–D). Because rescue with pantethine was most effective for dPANK/Fbl-depleted cells and pantethine was less toxic compared with CoA, our further analyses were focused on the rescuing potential of pantethine. The optimal effective concentration of pantethine for cells was 100 μM (Figure 2D).

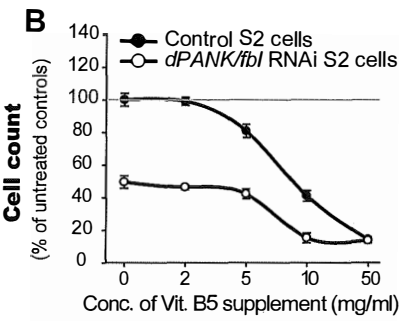
Pantethine rescues levels of CoA in dPANK/Fbl-depleted cells and in *dPANK/fbl* mutant flies

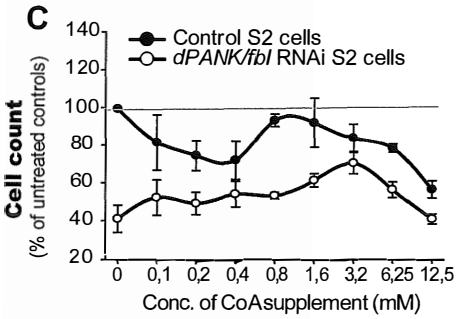
First, we tested whether addition of pantethine to the cell culture medium of dPANK/Fbl down-regulated cells could restore CoA levels. Indeed, a restoration of CoA was observed (Figure 2E). Next, we addressed the question whether pantethine addition to the food also rescues *dPANK/fbl* mutants. To identify the effective pantethine concentration in the fly food, various doses of pantethine were tested (Figure S3). The addition of pantethine at a concentration of 1.6 mg/mL induced a significant increase in climbing activity in *dPANK/fbl* mutants while inducing only a moderate side affect in wild types. This concentration was used in all further experiments unless indicated otherwise. Concomitantly, levels of CoA were restored upon feeding pantethine directly to *dPANK/fbl* mutants via the food (Figure 2F). These data suggested that there is a dPANK/Fbl-independent way to generate CoA from pantethine in both *Drosophila* S2 cells and in *Drosophila dPANK/fbl* mutant flies. Our data also indicated that pantethine provided via the food can still exert its CoA levels-restoring function.

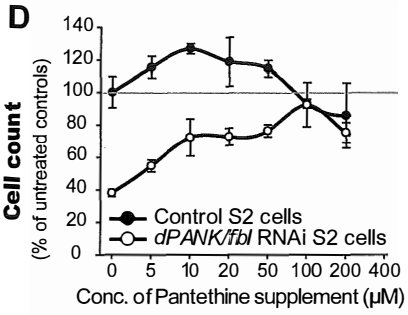
► Figure 2. Pantethine rescues cell count of dPANK/Fbl-depleted cells.

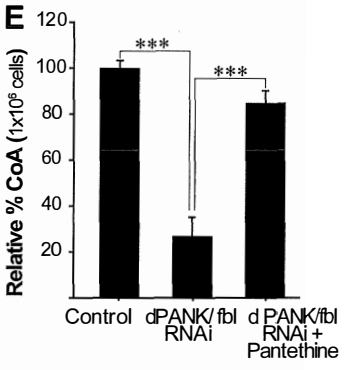
A, Control and *dPANK/fbl* RNAi-treated cells were counted and plated in equal numbers (0.35×10^6 cells/mL) 4 days after *dPANK/fbl* RNAi treatment, and proliferation was assayed by counting cells 3 days later. ***P < 0.001 (Student's t test). Error bars indicate SEM. Control cells and *dPANK/fbl* RNAi-treated cells were plated, and it was tested whether VitB5 (B), CoA (C), or pantethine (D) addition to the medium rescued the cell count of dPANK/Fbl down-regulated cells. 100% represents the number of control cells under normal culturing conditions. E, CoA levels were measured using HPLC in control cells and in *dPANK/fbl* RNAi-treated S2 cells (7 days after treatment) with and without addition of 100 μM pantethine. F, HPLC was used to measure CoA levels in wild types and *dPANK/fbl* mutants after supplementation of 1.6 mg/mL pantethine to the food. ***P < 0.001 (Student's t test). Error bars indicate SEM. b.d.l, below detection limit.

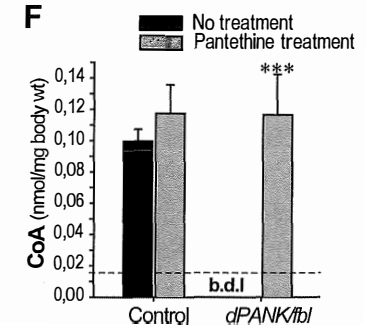

A
Cell count (1 X 10⁶)
***
Control
dPANK/fbl RNAi


B
Control S2 cells
dPANK/fbl RNAi S2 cells
Cell count
(% of untreated controls)
Conc. of Vit. B5 supplement (mg/ml)


C
Control S2 cells
dPANK/fbl RNAi S2 cells
Cell count
(% of untreated controls)
Conc. of CoA supplement (mM)


D
Control S2 cells
dPANK/fbl RNAi S2 cells
Cell count
(% of untreated controls)
Conc. of Pantethine supplement (µM)


E
Relative % CoA (1x10⁶ cells)
***
***
Control
dPANK/fbl RNAi
d PANK/fbl RNAi + Pantethine


F
No treatment
Pantethine treatment
CoA (nmol/mg body wt)
***
b.d.l
Control
dPANK/fbl

Mitochondrial structure and function are severely affected in *dPANK/fbl* mutants, and pantethine rescues these phenotypes

Mitochondrial dysfunction is associated with a number of neurodegenerative diseases [16, 17]. Several findings suggest that most likely PKAN is also a neurodegenerative disorder associated with impaired mitochondrial function; it has been shown that human PANK2 is localized in mitochondria [18], and that chemical inhibition of pantothenate kinase activity in primary hepatocytes induces abnormal mitochondrial morphology [19]. In addition, it was shown that a specific splice isoform of *Drosophila* PANK/fbl was localized in mitochondria of *Drosophila* S2 cells [10]. Together these data strongly suggest that human PANK2 and *Drosophila* dPANK/Fbl have a mitochondrial function and that impairment of pantothenate kinase might lead to mitochondrial abnormalities. To investigate this, mitochondrial morphology was examined. Flight muscles contain numerous densely packed mitochondria, and therefore this tissue was analyzed. Visual inspection of flight muscles with bright field microscopy revealed that the structure had a more "loose" appearance and contained more ruptures in *dPANK/fbl* mutants as compared with controls (Figure 3A). Moreover, the muscular degeneration was progressive with age in *dPANK/fbl* mutants. Electron microscopic analysis was performed for a more detailed analysis, and this revealed that, in contrast to wild types, mitochondria of *dPANK/fbl* mutants were severely affected. The mutant mitochondria were swollen and showed damaged cristae and ruptured membranes (Figure 3B). This analysis showed that low levels of CoA coincide with severely damaged mitochondrial structures in *dPANK/fbl* mutants. Pantethine feeding significantly reversed the morphological mitochondrial defects (Figure 3A and B).

In addition to this analysis, the more quantitative JC-1 assay (Figure S1 and SI Text) was used to measure the percentage of functional mitochondria. Under control conditions, 70.8% of S2 cells had functional mitochondria (Figure 3C). As a positive control for this assay, valinomycin was added to the media, and the percentage of cells with functional mitochondria dropped to 18% (Figure 3C). Under normal culturing conditions, in dPANK/Fbl down-regulated S2 cells, mitochondrial activity was less than 54% after 4 days of RNAi treatment and was less than 32% after 7 days of RNAi treatment compared with control cells (Figure 3C). Mitochondrial function was rescued to the levels of control cells after adding pantethine to the medium of dPANK/Fbl-depleted cells (Figure 3C). A similar assay was performed on isolated mitochondria from *Drosophila* flies. The results showed that *dPANK/fbl* mutants have 42% reduced mitochondrial function at day 6 as compared with control flies. Interestingly, feeding pantethine to *dPANK/fbl* mutants restored their mitochondrial function up to 84% of wild-type controls (Figure 3D).

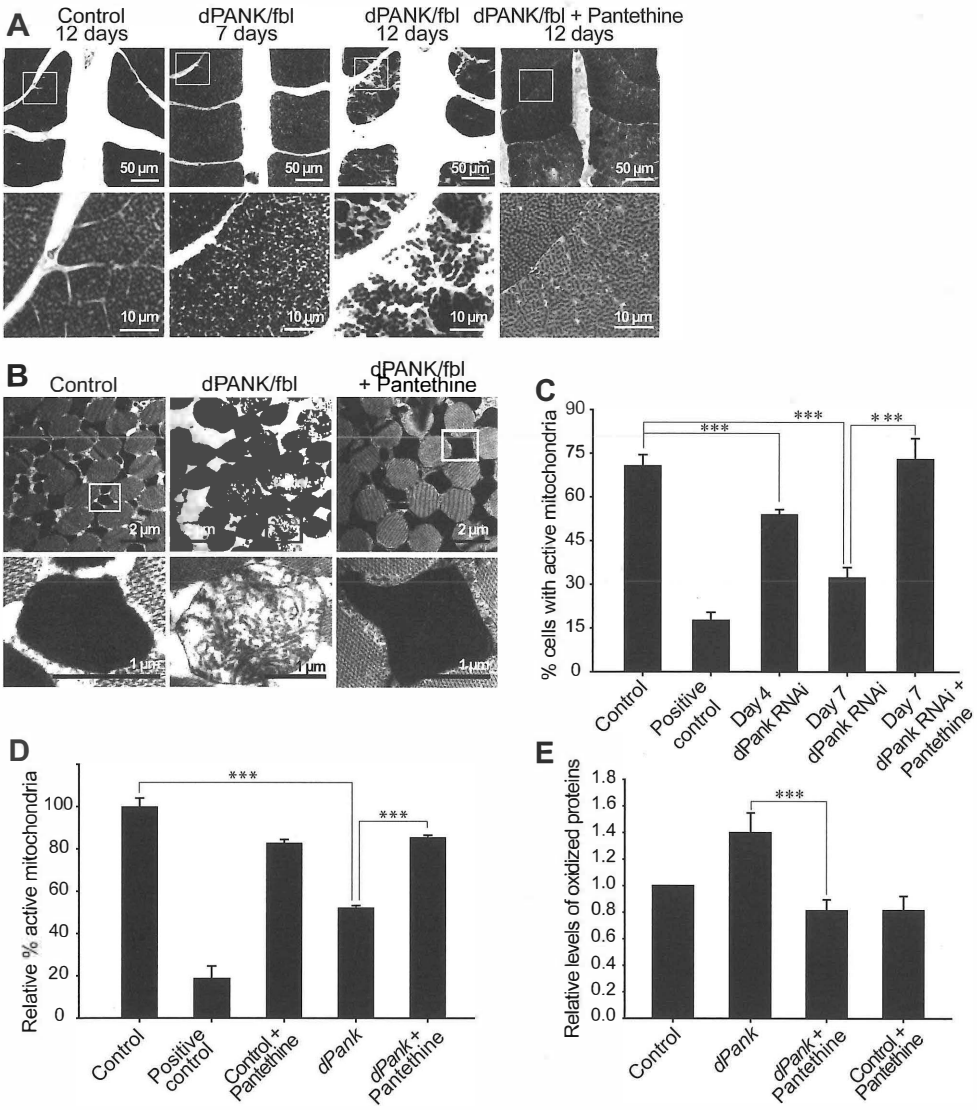
Pantethine reduces levels of increased oxidative damage of proteins in *dPANK/fbl* mutants

Previously, we showed that *Drosophila* CoA mutants displayed an increased sensitivity to oxidative stress [4]. Here we investigated whether pantethine was able to reduce increased levels of oxidative stress in *dPANK/fbl* mutants by using Oxyblot assays (Figure S4). Clearly, *dPANK/fbl* mutants showed increased levels of oxidative damage of proteins compared with wild type, and these levels were strongly reduced by addition of pantethine to the food (Figure 3E).

Pantethine improves locomotor abilities and rescues brain degeneration in *dPANK/fbl* mutants

So far, our results have demonstrated that pantethine restores CoA levels, improves mitochondrial function and reduces levels of oxidative damage in a *Drosophila* model for PKAN. Next, we investigated whether all these beneficial effects resulted in improvement of locomotor function in *dPANK/fbl* mutants. On average, wild-type larvae were able to crawl 50 cm in 9 min, whereas homozygous *dPANK/fbl* mutants reached only 20 cm (Figure 4A). Addition of pantethine to the larval food significantly improved larval crawling abilities, and an average distance of 30 cm was reached (Figure 4A). Although a strong improvement in larval crawling activity was observed, pantethine feeding was unable to completely rescue the mutant phenotypes. Our data were inconclusive as to whether incomplete rescue was because dPANK/Fbl has additional functions (other than the production of CoA) or whether pantethine has side effects that hampered complete recovery. The latter explanation was supported by our observation that, in wild types, crawling activity was also reduced after pantethine feeding (Figure 4A).

Previously, we demonstrated that *dPANK/fbl* flies showed reduced ability to climb at a young age [4]. Here we assayed whether this reduced ability to climb further deteriorates with age. Climbing tests of wild-type and homozygous *dPANK/fbl* flies at increasing age (2, 5, 10, 16, and 21 days), showed that mutants not only possessed impaired climbing abilities following eclosion but that they also experienced a steeper decline of the already-reduced climbing activity over time as compared with wild type (Figure 4B). Pantethine feeding significantly prevented the rapid decline of climbing ability of *dPANK/fbl* mutant flies (Figure 4B). Consistent with the data presented in Figure 4A, pantethine feeding induced a decrease in climbing activity in wild-type flies. In addition to these behavioral assays we tested a possible protective function of pantethine on neurodegeneration more directly by analyzing *dPANK/fbl* mutant brain tissue. *dPANK/fbl* mutants show increased numbers of vacuoles in their brains [4], indicating brain degeneration (Figure 4D). Pantethine also rescues this apparent neurodegenerative phenotype (Figure 4D and Figure S8).


A
Control
12 days
dPANK/fbl
7 days
dPANK/fbl
12 days
dPANK/fbl + Pantethine
12 days
50 µm
10 µm
B
Control
dPANK/fbl
dPANK/fbl
+ Pantethine
2 µm
1 µm
C
% cells with active mitochondria
90
75
60
45
30
15
0
***
***
***
Control
Positive control
Day 4 dPank RNAi
Day 7 dPank RNAi
Day 7 dPank RNAi + Pantethine
D
Relative % active mitochondria
100
80
60
40
20
0
***
***
Control
Positive control
Control + Pantethine
dPank
dPank + Pantethine
E
Relative levels of oxidized proteins
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
dPank
dPank + Pantethine
Control + Pantethine

◀ Figure 3. Impaired mitochondrial integrity and increased oxidative damage induced by disruption of dPANK/Fbl function is rescued by pantethine.

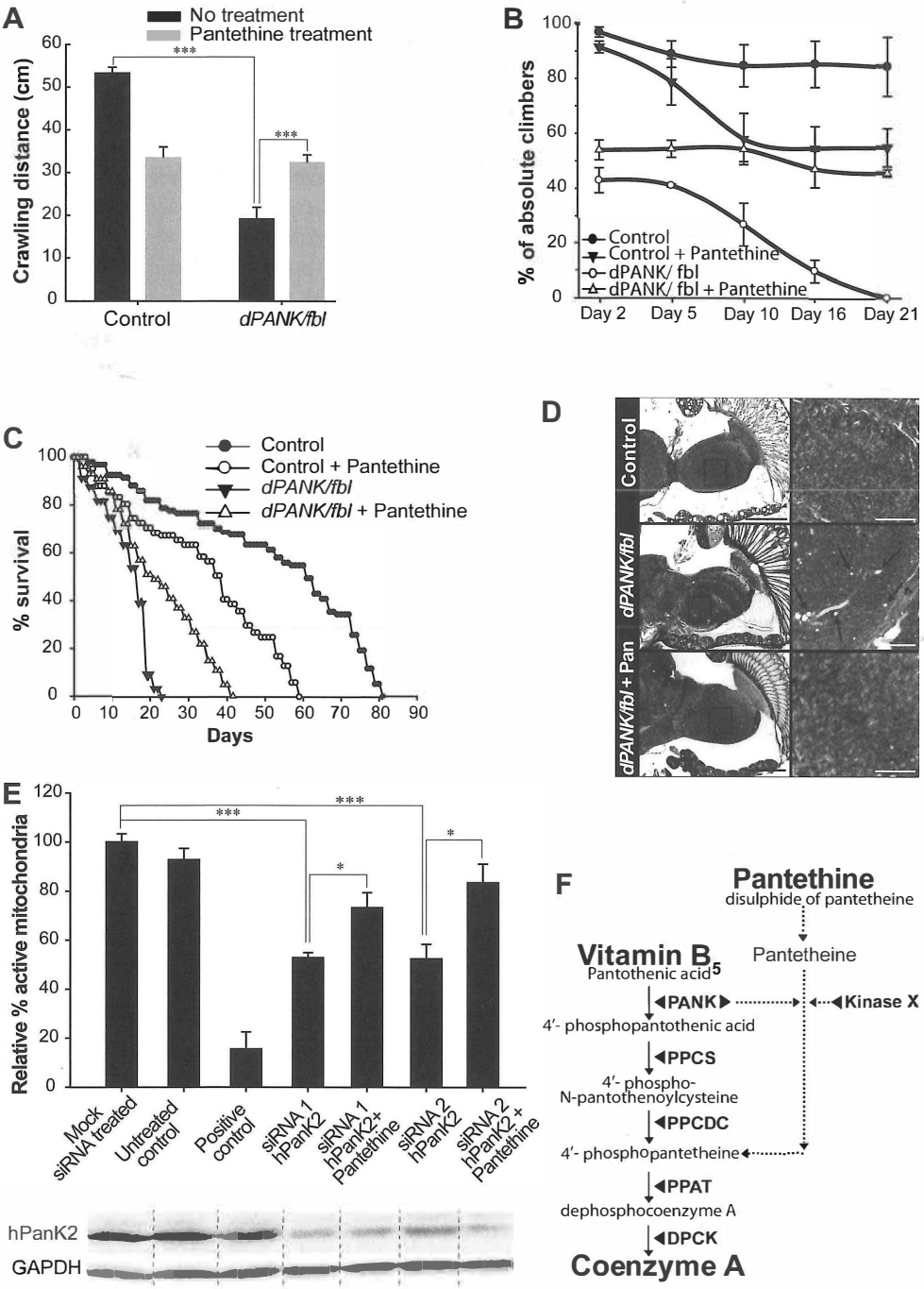
A, Morphological analysis of wild-type (12 days old) and *dPANK/fbl* mutant (7 days and 12 days old) flight muscles (untreated and treated with pantethine) was performed by light microscopy. B, Ultrastructural analysis of mitochondria in flight muscle of 12-day-old wild types and *dPANK/fbl* mutants (untreated and treated with pantethine). (Lower) Higher magnifications of the indicated areas. C - D, JC-1 assay was used to quantify mitochondrial function in control cells and in *dPANK/fbl* RNAi treated cells (C) in the absence and presence of pantethine and in *dPANK/fbl* mutants (D) in the absence and presence of pantethine. Valinomycin was used as a positive control. E, Oxyblots were used to measure levels of oxidative damage to proteins in *dPANK/fbl* mutants and the effect of supplementation of pantethine was investigated. ***P < 0.001 (Student's t test). Error bars indicate SEM.

Pantethine increases lifespan of *dPANK/fbl* mutants

Previously, we have demonstrated that *dPANK/fbl* mutants showed a severe reduction in lifespan [4]. We investigated whether rescue of all of the above-mentioned phenotypes with pantethine also coincides with increased lifespan of *dPANK/fbl* mutants. The maximal and median lifespan of *dPANK/fbl* mutants were 23 and 15 days, respectively (Figure 4C). Under these circumstances, wild-type flies showed maximal and median lifespans of 81 and 45 days, respectively (Figure 4C). Pantethine feeding increased the maximal lifespan of *dPANK/fbl* mutants from 23 days to 41 days and the median lifespan from 15 to 22 days (Figure 4C). Consistent with the data presented in Figure 4 A and B, pantethine feeding induced a reduction in lifespan in wild-type flies. Regardless of these deleterious side effects of pantethine in wild types, pantethine feeding clearly rescued various relevant abnormalities of *dPANK/fbl* mutants.

In mammalian HEK293 cells with down-regulated PANK2 levels, pantethine also improves mitochondrial function

Finally, we addressed the question of whether pantethine was also capable of rescuing an abnormal phenotype induced by impaired function of endogenous PANK2 in human cells, and for this we used mitochondria integrity as a read-out. First we tested whether down-regulation of PANK2 in HEK293 cells also results in decreased mitochondrial activity [quantified by the mitochondrial JC-1 assay (SI Text)]. Indeed, depletion of human PANK2 using two independent siRNAs resulted in decreased levels of PANK2 and decreased mitochondrial activity (Figure 4E). Addition of pantethine to the medium of PANK2-depleted cells resulted in a significant rescue of mitochondrial activity (Figure 4E). These data indicate that, also in human cells, pantethine was capable of protecting (albeit partly) against consequences of impaired PANK2 enzyme function.


A
No treatment
Pantethine treatment
Crawling distance (cm)
***
***
Control
dPANK/fbl
B
% of absolute climbers
Control
Control + Pantethine
dPANK/ fbl
dPANK/ fbl + Pantethine
Day 2
Day 5
Day 10
Day 16
Day 21
C
% survival
Control
Control + Pantethine
dPANK/fbl
dPANK/fbl + Pantethine
Days
D
Control
dPANK/fbl
dPANK/fbl + Pan
E
Relative % active mitochondria
Mock siRNA treated
Untreated control
Positive control
siRNA 1 hPanK2
siRNA 1 hPanK2+ Pantethine
siRNA 2 hPanK2
siRNA 2 hPanK2 + Pantethine
hPanK2
GAPDH
F
Pantethine
disulphide of pantetheine
Vitamin B5
Pantothenic acid
Pantetheine
PANK
Kinase X
4'- phosphopantothenic acid
PPCS
4'- phospho-
N-pantothenoylcysteine
PPCDC
4'- phosphopantetheine
PPAT
dephosphocoenzyme A
DPCK
Coenzyme A

◀ Figure 4. Pantethine is a protective compound in mutant flies and human cells.

A, Larval crawling abilities were measured in wild types and in *dPANK/fbl* mutants untreated and treated with pantethine. ***P < 0.001 (Student's t test). B, Percentage of climbers was measured in aging wild types and in aging *dPANK/fbl* mutants untreated and treated with pantethine. C, Cohorts of more than 120 flies (n = 3) of wild types and *dPANK/fbl* mutants were followed and survival curves were generated in the absence and presence of pantethine. All four curves were significantly different compared with each other (log-rank test, P < 0.001). D, Brain morphology was investigated at the light-microscopic level in (12-day-old) wildtypes, *dPANK/fbl* mutants untreated and treated with pantethine. (Left) Low magnification. (Bars, 100 µm.) (Right) Higher magnifications of boxed areas. (Bars, 20 µm.) Vacuoles are marked by arrows. E, Human HEK293 cells were treated with two independent siRNAs (siRNA 1 and siRNA 2) directed against human PANK2 mRNA. Western blot analysis with specific hPANK2 antibodies was used to investigate the effect of the RNAi treatment (48 h after RNAi treatment) on hPANK2 protein levels. GAPDH was used as a loading control. Addition of pantethine (100 µM) to the medium simultaneously with the RNAi treatment resulted in restoration of mitochondria function. Valinomycin was used as a positive control. ***P < 0.001; *P < 0.05 (Student's t test). Error bars indicate SEM. F, Scheme representing possible pathways for CoA biosynthesis from pantethine. Pantethine may be converted to pantetheine; pantetheine may be phosphorylated by a yet-unknown pantetheine kinase, other than pantothenate kinase (indicated by kinase X) Phosphorylated pantetheine (4'-phosphopantetheine) may enter the canonical *de novo* CoA biosynthesis pathway downstream of PPCDC and upstream of PPAT.

Discussion

In the current study, we used a *Drosophila* model for PKAN to investigate the consequences of impaired pantothenate kinase function and to identify possible protective compounds against the mutant phenotypes. We demonstrate that in *Drosophila dPANK/fbl* mutants and in dPANK/Fbl down-regulated S2 cells, CoA levels are significantly decreased. Low levels of CoA coincide with impaired mitochondrial integrity, increased levels of oxidized proteins, increased loss of locomotor function, neurodegeneration, and decreased lifespan. Our data are consistent with published data demonstrating that numerous neurodegenerative disorders are tightly linked to mitochondrial dysfunction and increased levels of oxidative stress [16, 17]. All of the *dPANK/fbl* phenotypes, including neurodegeneration, were more or less rescued by addition of the compound pantethine to the food. Our data support that the mechanism underlying pantethine protection in *dPANK/fbl* flies is specific and not general, because three other neurodegenerative (Parkinson's and two PolyQ) *Drosophila* models are not rescued by pantethine treatment (Figure S6). Pantethine has been already proved to be an effective treatment for hyperlipoproteinemia and dyslipidemia in human patients, and a dose of up to 1,200 mg pantethine per day for 24 weeks is tolerated without any side effects [20, 21]. Unfortunately it is currently unknown whether pantethine crosses the blood–brain barrier, although this knowledge is highly relevant to develop pantethine further as a possible treatment for PKAN.

For the first time, we show genetic evidence for the existence of a parallel pathway to the canonical *de novo* CoA biosynthesis starting from pantethine, which at least bypasses the first step of the pathway. We demonstrated that decreased levels of CoA were a clear consequence of impaired dPANK/Fbl function in *Drosophila*. Although this was an anticipated result, this consequence of impaired pantothenate kinase function has not been investigated in multicellular animals or in human patients. However, there are several reports that indirectly support our observations. Chemical inhibition of PanK1, PanK2, and PanK3 by HoPan in isolated murine hepatocytes resulted in a reduction of *de novo* CoA synthesis and reduction of total levels of CoA [19]. In Arabidopsis thaliana, it was demonstrated that mutations in several genes coding CoA biosynthesis enzymes resulted in impaired CoA biosynthesis and decreased levels of CoA [22-24]. Together, these and our data show that impaired function of CoA biosynthesis enzymes (including PANK) lead to a decreased rate of *de novo* CoA synthesis, and that normal *de novo* synthesis of CoA is required to maintain the physiological levels of CoA.

After establishing that CoA levels were indeed below detection in the *Drosophila* PKAN model, we demonstrated that pantethine is a very potent compound that can act as a starting substrate for generating CoA in a *dPANK/fbl* mutant background. How, exactly, pantethine can be converted into CoA is currently unclear. Classic biochemi-

cal studies using cell extracts showed that pantethine can be reduced into pantetheine [25, 26] and that this can be converted into 4′-phosphopantetheine [14]. The latter is an intermediate of the canonical *de novo* biosynthesis pathway and thus here, upstream from PPAT, the CoA *de novo* synthesis pathways starting from vitB5 and from pantethine may converge (Figure 4F). This is further supported by experiments showing that the decreased cell counts of dPPCS-depleted cells, but not of dPPAT-depleted cells, is rescued by pantethine (Figure S7). Possible enzymes that catalyze the phosphorylation of pantetheine have never been genetically identified. However, it has been shown that specific purified enzyme preparations were able to phosphorylate both VitB5 and pantethine with similar kinetics [13]. Based on these studies it was assumed, but never tested, that pantothenate kinase is the only enzyme present that can phosphorylate pantetheine. However, these earlier studies did not exclude the presence of additional kinases (other than pantothenate kinase) in the purified enzyme preparations with pantetheine kinase activities. It is also possible that pantethine can be converted into CoA not via 4′-phosphopantetheine but via a completely alternative route. Regardless of the exact route, our data suggest that this pathway can most likely occur independently from pantothenate kinase based on the following. (i) In both *dPANK/fbl* mutants and in *dPANK/fbl* down-regulated cells, the levels of *dPANK/fbl* are severely reduced and it is unlikely that these reduced *dPANK/fbl* protein levels are responsible for the restoration of CoA levels after pantethine addition. (ii) If some residual pantothenate kinase activity were present in *dPANK/fbl* mutants and in dPANK/Fbl down-regulated cells, addition of extra VitB5 should have been beneficial also. However, addition of extra vitB5 did not lead to rescue in *dPANK/fbl* (Figure S5) mutants and in dPANK/Fbl down-regulated cells (Figure 2B). Thus all of the above results suggest the presence of an alternative route for *de novo* synthesis of CoA independent from pantothenate kinase. Apart from whether residual activity of pantothenate kinase is required for pantethine rescue, our findings are still relevant for PKAN-related research because most of the patients with *PANK2* gene mutations still have some residual activity of pantothenate kinase [18].

Regardless of the mechanisms behind pantethine toxicity in wild-type cells, the exact pathway of pantethine conversion into CoA, and whether residual activity of pantothenate kinase is required for pantethine rescue, our data at least suggest the existence of an alternative pathway that uses pantethine as a primary compound to generate *de novo* CoA in the presence of impaired pantothenate kinase function. This knowledge allows the development of a possible future therapy for PKAN.

Materials and Methods

Drosophila strains - The *Drosophila* strain y1w1118 was used as a wild-type control (Bloomington Stock Centre). The hypomorphic *dPANK/fbl*1 mutant flies were used for all assays [2, 4].

Pantethine supplementation - D-Pantethine (Sigma) was added at a concentration of 1.6 mg/mL in standard fly food; 100 μM of D-Pantethine was added to S2 cell and HEK293 medium, except where mentioned otherwise.

Physiological assays - To study larval crawling, late third instar homozygous *dPANK/fbl* larvae were placed on 1% nonnutritive agar in a Petri dish. Total distance crawled by the larvae during 9 min was measured. To study the adult lifespan, newly eclosed flies (n > 100, 1 or 2 days old) were collected and raised on standard medium at 25 °C in a dry Petri dish with food (2.29 cm2; with or without pantethine) at the center of the Petri dish. The number of dead flies was counted every 2 days. Each experiment was repeated three times. For a climbing assay, adult flies were used to investigate climbing performance as previously described [27]. The experiment was repeated three times (n > 100).

CoA measurements - CoA levels were measured from fly extracts (100 flies, 6 days old) or from *Drosophila* Schneider's S2 cells using HPLC (sample preparation and HPLC analysis are described in SI Text). C

Cell culture and PANK knockdown - *Drosophila* Schneider's S2 Cells were cultured, and RNAi knockdown of dPANK/Fbl was performed as previously described [28] (SI Text). Mammalian cell culture and siRNA knockdown of hPANK2 are described in SI Text.

Electron and light microscopy - Flies were immersed in fixative solution (2.5% glutaraldehyde in 0.1 M cacodylate, pH7.8). Postfixation was performed in 2% OsO4 for 2 h at 4 °C. Dehydration was carried out with graded ethanol series followed by a propylene wash and preembedding in (1:1) propylene:epon solution. Embedding of the flies was performed in EPON. For light microscopy, sections (1–2 μm) were cut using a Reichert Ultracut microtome and stained with Toluidine Blue. For ultrastructural analysis of mitochondria, thin sections (60 nm) were cut from the same samples and analyzed by electron microscopy.

Mitochondrial assays - Mitochondria were isolated from 7-day-old flies as previously described [29]. For measurement of mitochondrial membrane potential, J-aggregate-forming lipophilic cation (JC-1) was used to evaluate mitochondrial damage [30]. The JC-1 assay (Sigma) was performed according to the manufacturer's manual (SI Text and Fig. S1).

Protein oxidation detection - Protein lysates were prepared in RIPA buffer containing 2% β-mercaptoethanol. Total protein solutions were incubated with 2,4-dinitrophenylhydrazine (DNP) according to the OxyBlot protein oxidation detection Kit (Chemicon). The total amount of oxidized proteins was quantified for each sample by measuring chemiluminescence from the whole lane and oxidized protein levels were normalized using β-actin as a loading control (Fig. S4).

Antibodies - dPANK/Fbl (1:4,000) [4], hPANK2 (1:2,000; a gift from J. Gitschier, University of California–San Francisco), GAPDH (1:10,000; Fitzgerald Industries), β-actin, and γ-tubulin (Sigma) were used. HRP-conjugated antimouse or antirabbit antibodies were used (1:2,000; Amersham) as secondary antibodies.

Acknowledgments

We thank Floris Bosveld and Harm Kampinga for stimulating discussions. This work was supported by a VIDI grant (to O.S.), a Neurodegeneration with Brain Iron Accumulation Disorders Association grant (to O.S and S.H.), and a Topmaster grant from the Graduate School GUIDE (to A.R.).

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Supplementary Methods

Evaluation of functional mitochondria using a JC-1 assay - Mitochondrial function can be quantitatively assessed by measuring changes in the mitochondria transmembrane potential using JC-1 which is J-aggregate-forming lipophilic cationic fluorochrome (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (Sigma) assay (1). At high mitochondrial membrane potentials, JC-1 accumulates in the mitochondria and forms J-aggregates that show a red fluorescence emission at 590 nm. At lower mitochondrial potentials, less dye enters mitochondria, resulting in monomers that show green fluorescence emission at 530 nm. By using this assay, one can quantify highly active mitochondria (with both red and green fluorescence) and depolarized mitochondria (with green fluorescence only). In addition, by using this assay, it is possible to investigate the mitochondria integrity of suspension cells, of attached cells and to investigate integrity of isolated mitochondria from tissues. For these assays, valinomycin is added as a control, because this K+ ionophor depolarizes the mitochondrial membrane and induces a sharp decrease in red fluorescence emission at 590 nm, representing an increase in impaired mitochondria integrity.

Mitochondrial potential in *Drosophila* S2 cells was estimated using the flow cytometer analysis method for JC-1 probe (Molecular Probes protocol: MitoProbe JC-1 assay kit for Flow Cytometry (M34152). Briefly: 1 × 106 cells were suspended in 500 µL of growth medium and incubated with valinomycin or left untreated. Cells were then centrifuged at 400 rpm (5 min at 4 °C) and resuspended in JC-1 solution buffer (10 µg/mL). After incubation (15 min), cells were pelleted, resuspended in ice-cold PBS, and analyzed immediately using a flow cytometer. Monomers and J-aggregates of JC-1 were simultaneously excited using 488-nm laser, and emission was quantified in FL1 (530 nm) and FL2 (590 nm) channels. Mitochondria containing red JC-1 aggregates (mitochondria with a normal membrane potential; active mitochondria) from viable cells were detectable in FL2 channel, and green JC-1 monomers (mitochondria with a depolarized membrane; impaired mitochondria) were detectable in FL1 channel (Figure S1). The results were plotted as the percentage of cell with active mitochondria (FL2) from total number of cells analyzed (20,000 cells per analysis) (Figure 3C).

Analysis of mitochondria isolated from flies was performed according to the manufacturer's protocol (Sigma; mitochondrial isolation Kit). In short: 100 µL of the JC-1 Staining Solution was added to 10 µL of isolated mitochondria resuspended in mitochondrial maintenance medium (Sigma; mitochondrial isolation kit) in a 96-well plate. Fluorescence was measured in a spectrofluorometer (FL600 Biotek) using the following settings: excitation wavelength, 490 nm; emission wavelength, 590 nm. Fluorescence produced (FLU) per well was recorded, and total FLU per milligram of proteins (FLU/mgP) was calculated. FLU/mgP is an indication of the amount of J-aggregate formation

and a measurement of active mitochondria. In control cells this was set to 100%. The amount of FLU/mgP was indicated for every condition as a percentage of the FLU/mgP in control cells (Figure 3D). Analysis of mitochondria in HEK293 cells was performed according to protocols for adherent cells (2, 3). In short, adherent cells were incubated with JC-1 solution (10 μg/mL) in growth medium for 15 min, washed twice with ice-cold PBS, and fluorescence measured in a spectrofluorometer. To normalize for the amount of cells, the ratio of FLU for active mitochondria (590 nm alone) to the total FLU from the well (sum of 530 nm and 590 nm) was calculated, and in control cells this was set to 100% (Figure 4D).

Measurement of CoA levels by HPLC - For fly sample preparation, homozygous *dPANK/fbl* flies (6 days old), 60 female and 40 males per experiment, were collected and weighed. Flies were then snapfrozen in Liquid N2, and 200 μL of solvent buffer (5% sulfosalicylic acid containing 50 μM DDT) was added. After thorough grinding, the samples were sonicated three times for 10 s on ice. Samples were centrifuged and supernatant was collected for HPLC analysis of CoA. Before analysis, 2 μL of Ammonia (25%) was added to 98 μL of the sample solutions. For *Drosophila* Schneider's S2 cell sample preparation, cells were pelleted, and 200 μL of solvent buffer (5% sulfosalicylic acid containing 50 μMDDT) was added. Samples were sonicated and centrifuged, and the supernatant was collected for HPLC analysis of CoA. Before analysis, 2 μL of ammonia (25%) was added to 98 μL of the sample solutions.

CoA was measured according to a slightly modified, previously described method (4) using HPLC. A Nucleosil 120 C18 (4.6 × 150 mm, 3-μm) column was used, together with an Agilent Technologies Guard column C18 (4.6×12.5mm, 5 μm), with an injection volume of 30 μL per sample. Mobile phase A consisted of 100mmol/L sodium dihydrogen phosphate and 75 mmol/L sodium acetate. The pH of the buffer was set at 4.6 with phosphoric acid. Mobile phase B consisted of 30% methanol and mobile phase A 70%. The temperature of the column was maintained at 35°C. The solvent gradient consisted of 10–40 % B in 10 min and 40–90 % in 8min. The column was equilibrated with 10% B between each sample analysis. The flow rate was maintained at 1.2 mL/min. HPLC analysis was performed using a Shimadzu-VP system (Shimadzu).

Drosophila Schneider's S2 cell culture and RNAi - For generation of the dsRNA the following primers were used:

Gene	Primer
dPANK/Fbl	fwd-CGTGATACGCACCTACAGATG
	rev-GCCATTGGACCAGAACTCCAT
dPPCS	fwd-GGCACAACAAGCTCCAGAAT
	rev-CTTGCGTGTCTGCAGCACAT
dPPAT	I) fwd-GCGAGCCATCGAGAAGTACG
	rev-CCGAGTCATCCAGGAAGATTGT
dPPAT	II) fwd-GCCCACGTGATCGACTGCGAT
	rev-CCACTTCGCTCAACTTGTTGC

As a control, non-relevant (human gene; hMAZ) dsRNA was used. dsRNA was produced and purified with MEGAscript RNAi Kit (Ambion) according to the manufacturer's instructions. Down-regulation of dPANK/Fbl protein was investigated by immunoblotting using dPANK/Fbl specific antibodies for every individual experiment (Figure S2).

Mammalian cell culture and siRNA knockdown of hPANK2 - PANK2 knockdown in HEK293 cells was performed under conditions of regulated levels of pantothenic acid (vitB5) using custom made vitB5 free DMEM (ThermoScientific) supplemented with 0.4 mg/L vitB5 (Sigma), 10 % dialyzed serum (Gibco), 100 U/mL penicillin, and 100µg/mL streptomycin (Invitrogen). Two different human PANK2-specific small interfering RNAs (siRNAs) were used: siRNA1 (Dharmacon; D-003797-04) and siRNA2 (Ambion; AM51321). Nonsilencing control siRNA was purchased from Dharmacon (VOSMC 000005). HEK293 cells were transfected with 100 nM siRNA using siPORT Amine transfecting agent (Ambion) according to the manufacturer's protocol. Knockdown efficiency was assayed by immunoblotting 48 h after transfection.

RT-PCR Analysis - Total RNA was extracted from the S2 cells using the Absolutely RNA kit (Stratagene). A 1-μg quantity of total RNA was used for cDNA transcription (Invitrogen). Semi-quantitative PCR was performed using the following primers to amplify parts of rp49, dPPCS, and dPPAT (product was amplified for 21, 25, and 29 cycles):

Gene	Primer
dPPCS	fwd- ACTTCACCGGCCAGCAGTTC
	rev- AATCGTCGGCGCTCCATCTC
dPPAT	fwd-GCGAGCCATCGAGAAGTACG
	rev-CCGAGTCATCCAGGAAGATTGT
rp49	fwd-GCACCAAGCACTTCATCC
	rev CGATCTCGCCGCAGTAAA

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Supplementary Figures

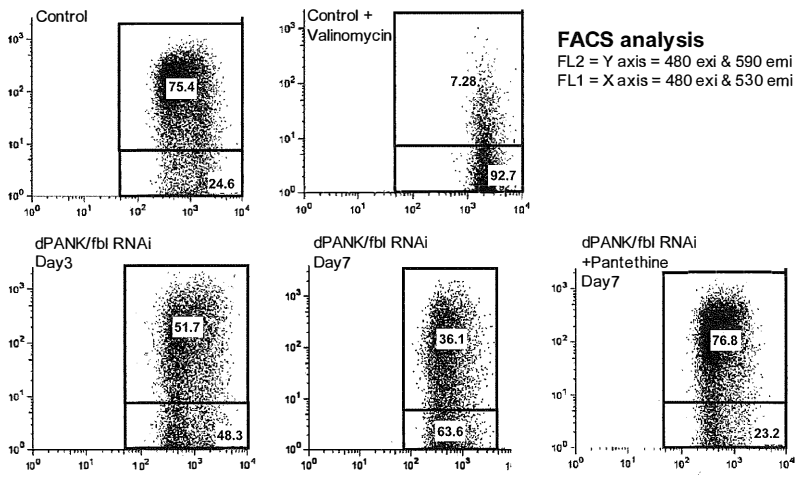


Figure S1. Analysis of functional mitochondria in dPANK/Fbl depleted cells by FACS analysis.

FACS analysis in combination with a JC-1 assay was used to measure changes in the mitochondria transmembrane potential, and this enabled the quantification of active mitochondria (detailed description of JC-1 assay described above in SI Text). Dot plots are shown for the following conditions: control cells; control cells treated with Valinomycin; dPANK/Fbl depleted cells (untreated and treated with pantethine). Upper boxed areas represent cells with a red and green fluorescence emission of 590 nm and 530 nm above a specific threshold, representing cells with active mitochondria. Lower boxed areas represent cells with a red fluorescence emission of 590 nm below a specific threshold (and with a green fluorescence emission of 530 nm above a specific threshold) representing cells with a disturbed mitochondrial membrane potential. Percentages of cells are indicated in boxed areas.

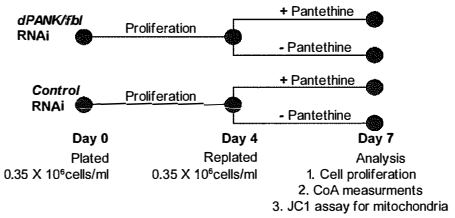


Figure S2. Schematic representation of RNAi experiments

At day 0, cells were plated in equal densities and were treated with *dPANK/fbl* dsRNA or with control dsRNA. After 4 days, cells were replated in equal densities and left further untreated or were treated with pantethine. On day 7, various assays were performed. Down-regulation induced by the RNAi treatment for all assays was measured with Western blot analysis using dPANK/Fbl antibodies.

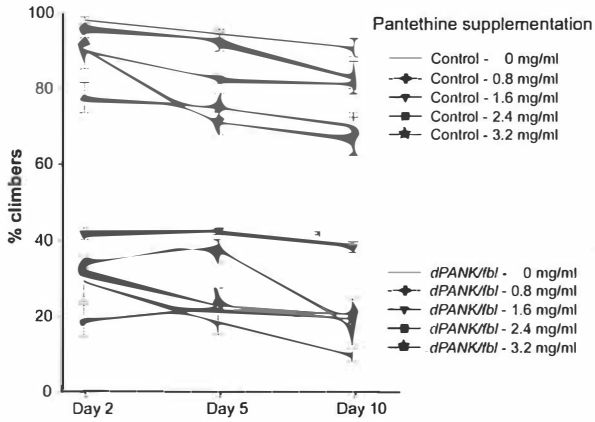


Figure S3. A 1.6-mg quantity of pantethine per milliliter of food is the optimal concentration to rescue climbing ability

Various concentrations (0.8 mg/mL, 1.6 mg/mL, 2.4 mg/mL, and 3.2 mg/mL) of pantethine were added to the food of wild-type flies and *dPANK/fbl* mutants. Pantethine was added immediately after eclosion, and the food was refreshed every day. On days 2, 5, and 10, climbing activity was measured. Adding 1.6 mg of pantethine per milliliter of food induced a significant rescue of climbing activity. Moreover 1.6 mg pantethine showed only a mildly reduction of climbing activity in wild-type flies compared with 2.4 mg pantethine. Based on these results, 1.6 mg pantethine per milliliter of food was used for the experiments described in this article.

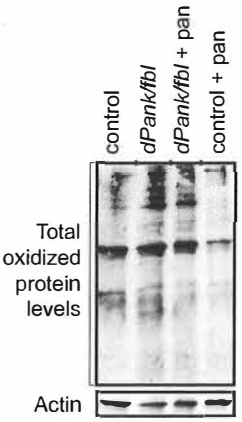


Figure S4. *dPANK/fbl* flies have increased levels of oxidized proteins that are rescued upon pantethine feeding.

Oxyblot analysis revealed that *dPANK/fbl* mutants have higher levels of total oxidized proteins as compared with wild type. Daily feeding of pantethine for 6 days immediately after eclosion results in reduction of oxidative damage to the proteins in *dPANK/fbl* mutants. Actin is used as loading control.

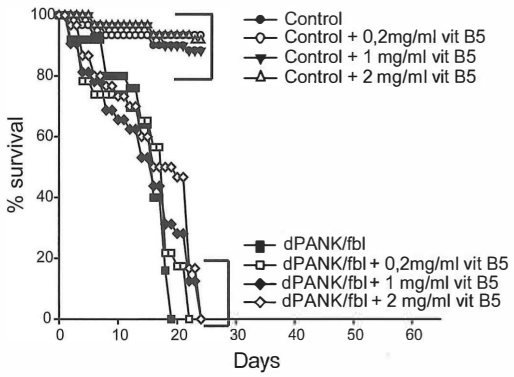
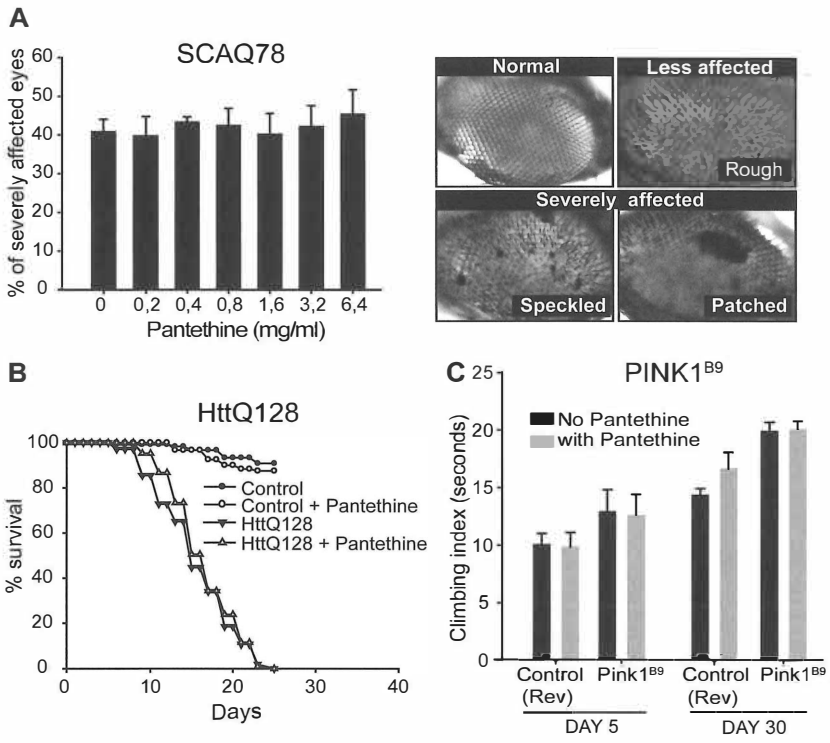


Figure S5. Vitamin B5 does not increase the lifespan of *dPANK/fbl* mutants

To investigate the effect of vitamin B5, various concentrations (0.2 mg/mL, 1 mg/mL, and 2 mg/mL) of vitamin B5 were added to the food and tested for their potential to increase lifespan. At 22 days after feeding the various concentrations of vitamin B5, all *dPANK/fbl* mutants had died, and no significant increase in lifespan was observed.



◀ Figure S6. *Drosophila* models for the neurodegenerative diseases: Spinocerebellar Ataxia-type 3 (SCA-3), Huntington, and Parkinson are not rescued by pantethine.

A, Flies expressing a truncated form of Ataxin 3 protein containing an expanded repeat of 78 glutamines in eyes show a rough-eye-phenotype (1) and are referred to as SCA3Q78 flies. This transgenic *Drosophila* strain is a model for SCA3 and has been used to investigate modifiers of toxicity induced by polyglutamine in SCA3 related neurodegeneration. Eye abnormalities are classified as "rough" or as "severely affected" as previously described (2). Protective compounds will reduce the percentage of severely affected eyes. Increasing concentrations of pantethine did not result in a significant decrease of the percentage of severely affected eyes (>250 eyes were scored for each condition). B, Flies expressing a truncated form of Huntingtin containing an expanded repeat of 128 glutamines show a neurodegenerative phenotype including a reduced lifespan of 24 days and are referred to as HttQ128 flies (3). Addition of (1.6 mg/mL) pantethine to the food did not increase the lifespan of HttQ128 flies. For each condition, more than 100 flies were used. C, Mutations in human
PINK1 are linked to parkinsonism. The *Drosophila* PINK1 gene is an ortholog of the human PINK1 gene, and *Drosophila* PINK1B9 mutants show a progressive impairment to climb as they age (4). Addition of (1.6 mg/mL) pantethine to the food did not improve the climbing ability of PINK1B9 mutants. For each time point, >100 flies were used. As a control, PINK1B9 revertants were used that overexpress the wild-type *Drosophila* PINK1 gene in the PINK1B9 mutant background. Climbing index is defined as the average climbing time required to climb 15 cm by 50% of the flies (4).

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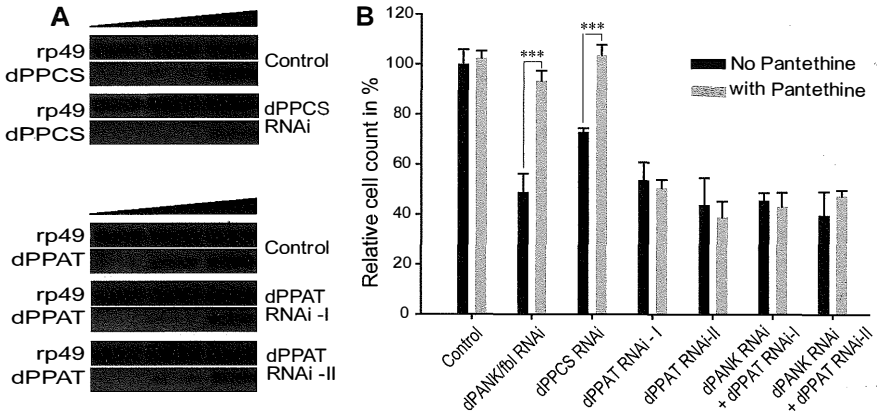

Figure S7. Pantethine rescues cell count of dPPCS-depleted cells but not of dPPAT-depleted cells.

RNAi was used to down-regulate dPPCS, dPPAT, or dPANK/Fbl and dPPAT simultaneously in *Drosophila* S2 cells. To down-regulate dPPAT, two independent non-overlapping RNAi constructs were used (detailed description of constructs is provided above in SI Text). Down-regulation induced by the RNAi treatment of dPPCS and dPPAT was investigated by RT-PCR. Down-regulation of dPANK/Fbl induced by RNAi treatment was controlled by using Western blotting (as in Fig. 1C). A, PCR products revealed a significant down-regulation of dPPCS and dPPAT mRNA after RNAi treatment. B, In dPANK/Fbl-depleted cells, dPPCS-depleted cells, dPPAT-depleted cells, and dPANK/Fbl-dPPAT-doubledepleted cells, the cell count was decreased as compared with control cells. Pantethine addition to the cell culture medium significantly increased the cell count of dPANK/Fbl-depleted cells and of dPPCS-depleted cells but not the cell count of dPPAT-depleted cells and of dPANK/Fbl-dPPAT-double-depleted cells. These data strongly suggest that dPPAT is required for the pantethine rescue of dPANK/Fbl-depleted cells. ***P < 0.001 (Student's t test). Error bars indicate SEM.

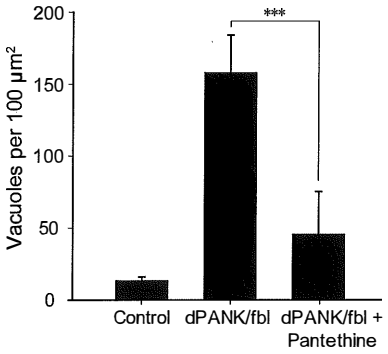

Figure S8. Amount of brain vacuoles in *dPANK/fbl* mutant flies is decreased after pantethine treatment

Number of vacuoles in the brain region (indicated in Figure 4D) was measure by ImageJ software (http://rsb.info.nih.gov/ij/index.html). The method of quantification is outlined in the ImageJ documentation ("Particle Analysis"). The total amount of vacuoles was calculated per 100 µm2 from comparable regions (indicated in the boxed areas in Figure 4D). For every condition, four brains were examined of 12-day-old flies. After eclosion, flies were kept on standard food or on standard food supplemented with 1.6 mg/mL pantethine. ***P < 0.001 (Student's t test). Error bars indicate SEM.

Impaired Coenzyme A metabolism affects histone and tubulin acetylation in *Drosophila* and human cell models of pantothenate kinase-associated neurodegeneration

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EMBO Mol Med. 2011
doi: 10.1002/emmm.201100180

Abstract

PKAN is a neurodegenerative disease with unresolved pathophysiology. Previously, we observed reduced Coenzyme A levels in a *Drosophila* model for PKAN. Coenzyme A is required for acetyl-Coenzyme A synthesis and acyl groups from the latter are transferred to lysine residues of proteins, in a reaction regulated by acetyltransferases. The tight balance between acetyltransferases and their antagonistic counterparts histone deacetylases is a well-known determining factor for the acetylation status of proteins. However, the influence of Coenzyme A levels on protein acetylation is unknown. Here we investigate whether decreased levels of the central metabolite Coenzyme A induce alterations in protein acetylation and whether this correlates with specific phenotypes of PKAN models. We show that in various organisms proper Coenzyme A metabolism is required for maintenance of histone- and tubulin acetylation, and decreased acetylation of these proteins is associated with an impaired DNA damage response, decreased locomotor function and decreased survival. Decreased protein acetylation and the concurrent phenotypes are partly rescued by pantethine and HDAC inhibitors, suggesting possible directions for future PKAN therapy development.

Introduction

Recently a large body of evidence has emerged to suggest that protein acetylation plays an important role in key cellular processes [1]. The balance between Histone or K-acetyltransferases (HATS or KATS) and histone deacetylases (HDACs) is well-documented as influential in the homeostasis of protein acetylation [2-4]. Acetyl-Coenzyme A (Acetyl-CoA) is the source for the acyl group that is transferred to lysine residues, and it was demonstrated that down-regulation of enzymes required for the synthesis of acetyl-CoA induce reduction in acetylation of specific proteins [5-7]. However, it is completely unknown whether levels of metabolites themselves influence protein acetylation. Considering that CoA is a central metabolic cofactor involved in over 100 metabolic reactions [8] and is also required to synthesize acetyl-CoA from citrate or acetate, CoA is an interesting candidate-metabolite with potential influence over protein acetylation. Remarkably, a possible role of CoA metabolism on protein acetylation has never been directly investigated and it is unclear whether or not protein acetylation levels respond to decreasing concentrations of cellular CoA, or whether increased activity of HATS/KATS compensate for decreased levels of Coenzyme A.

The *de novo* biosynthesis route of CoA is a well conserved enzymatic pathway. The first and rate-limiting step, the phosphorylation of vitamin B5, is catalyzed by the enzyme pantothenate kinase (PANK) [8] (Figure 1A). The CoA biosynthesis pathway has received renewed attention after the discovery that mutations in the human *PANK2* gene are associated with the severe neurodegenerative disease Pantothenate Kinase-Associated Neurodegeneration (PKAN) [9]. The pathology of PKAN is complex, and exactly how impaired *de novo* biosynthesis of CoA is linked to neurodegeneration as in PKAN is largely unknown [10].

In *Drosophila melanogaster* a pantothenate kinase ortholog is present and referred to as dPANK/fumble [11, 12]. *dPANK/fbl Drosophila* mutants possess a neurodegenerative phenotype and a greatly reduced life span. We recently showed that down-regulation of the enzyme pantothenate kinase (dPANK/Fbl) in flies and cultured cells results in decreased levels of total CoA, and further that addition of the compound pantethine to the food restored CoA levels and rescued the mutant phenotype [12, 13]. This model can now be used to further manipulate and measure CoA levels and to study directly the biological consequences of decreased CoA levels and to understand the molecular mechanisms underlying PKAN. The neurodegenerative *Drosophila* PKAN model is further characterized by increased sensitivity to DNA damage, an explanation for which is currently lacking. Here we exploited this *Drosophila* model to investigate 1) whether or not decreased CoA levels affect the acetylation levels of specific proteins and 2) if so, whether this abnormal acetylation status of specific proteins coincide with the pleiotropic phenotype of the *Drosophila* model for PKAN and 3) if so, whether restoration of acetylation levels of specific proteins can rescue apparent PKAN-related phenotypes in *Drosophila*.

We show that when CoA levels are reduced, there is no compensatory mechanism to maintain the normal acetylation levels of histones and tubulin, and decreased acetylation of these proteins is associated with key characteristics of a *Drosophila* model for PKAN. After feeding pantethine or HDAC inhibitors, acetylation levels of histones and tubulin are restored and this coincides with improved viability, locomotor function and survival after DNA damaging insults. Additionally, we demonstrate that the correlation between pantothenate kinase activity and acetylation of specific proteins is conserved among species and thus decreased protein acetylation may underlay the pathogenesis of PKAN.

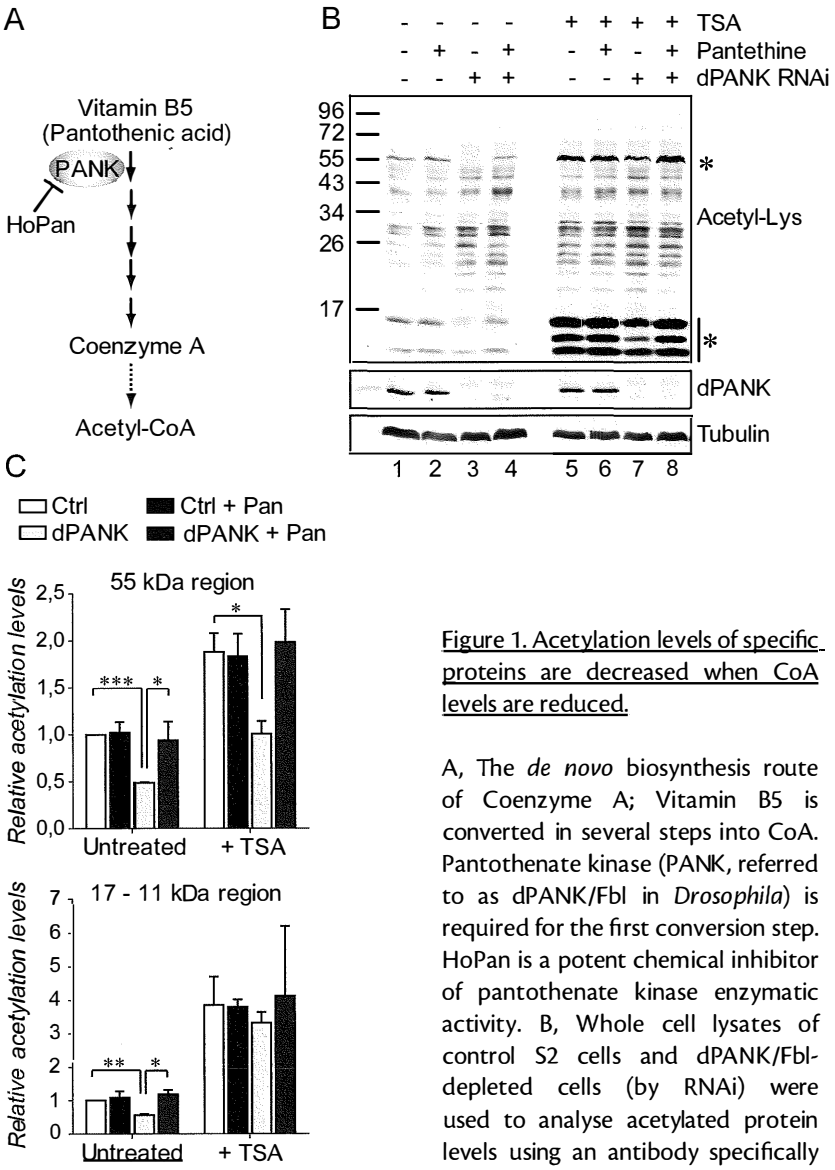
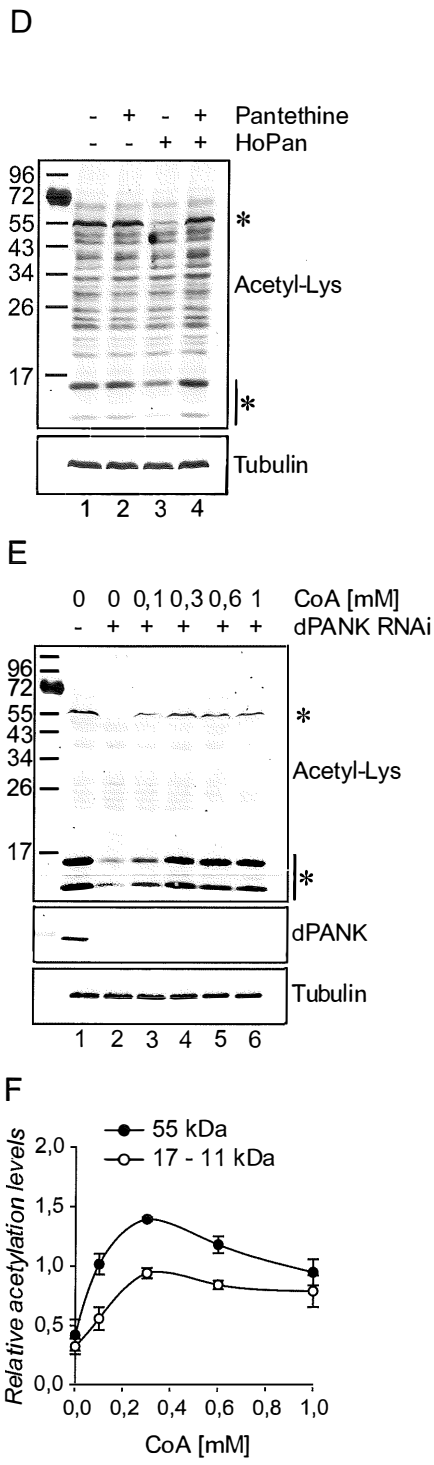


Figure 1. Acetylation levels of specific proteins are decreased when CoA levels are reduced.

A, The *de novo* biosynthesis route of Coenzyme A; Vitamin B5 is converted in several steps into CoA. Pantothenate kinase (PANK, referred to as dPANK/Fbl in *Drosophila*) is required for the first conversion step. HoPan is a potent chemical inhibitor of pantothenate kinase enzymatic activity. B, Whole cell lysates of control S2 cells and dPANK/Fbl-depleted cells (by RNAi) were used to analyse acetylated protein levels using an antibody specifically



recognizing acetylated-lysine residues. Control cells and dPANK/Fbl-depleted cells were left untreated or treated with pantethine (0,1 mM), with TSA or with TSA and pantethine. C, Quantification of the relative levels of acetylation compared to control cells for the indicated band of 55 kD in size and quantification of the relative levels of acetylation for the indicated bands around 17-11 kD. D, S2 cells were incubated with 0,5 mM HoPan, and/or with 0,1 mM pantethine. Whole cell extracts were probed with anti-acetyl-lysine antibody. E) dPANK/Fbl- depleted cells were left untreated or increasing concentrations of CoA where added to the cell culture medium. Whole cell extracts were probed with an acetyl-lysine antibody. F, Quantification of the relative levels of acetylation compared to control cells after addition of various concentrations of CoA for the indicated band of 55 kD in size and quantification of the relative levels of acetylation for the indicated bands around 17-11 kD. Asterisks indicate bands which show a decreased signal in the dPANK/Fbl depleted or HoPan treated cells. Efficiency of the RNAi treatment was controlled by using an antibody specifically recognizing dPANK/Fbl [12]. As a loading control tubulin was used.

Results

CoA levels can be modified and measured and a decrease in CoA leads to decreased acetylation of specific proteins

To investigate the influence of CoA levels on protein acetylation, RNAi was used to down-regulate dPANK/Fbl protein in *Drosophila* Schneider's S2 cells (Figure 1B). Levels of total CoA are severely reduced under these circumstances [13]. To determine the general acetylation status of proteins under these conditions, immunoblots of whole cell extracts (from control and dPANK/Fbl-depleted cells) were incubated with an antibody specifically recognizing acetylated-lysine. In dPANK/Fbl-depleted cells the levels of some specific proteins (indicated by asterisks, Figure 1B), between 17-11 kD and 55 kD in size, appeared to be reduced as compared to control cells (compare lane 1 and lane 3, for quantification see Figure 1C). Only the acetylation levels of specific proteins and not all proteins recognized by the anti-acetyl-lysine antibody were affected under circumstances of decreased CoA levels. Previously, we demonstrated that addition of the compound pantethine restored CoA levels in a dPANK/Fbl-depleted background via a (yet unresolved) non-canonical CoA *de novo* biosynthesis pathway [13]. Addition of pantethine reversed the acetylation levels of the indicated proteins back to wildtype (compare lane 3 and 4, Figure 1B-C), indicating that altered acetylation of the indicated proteins coincides with decreased levels of CoA and not with decreased levels of the dPANK/Fbl enzyme. Moreover, inhibition of pantothenate kinase activity by the selective chemical pantothenate kinase inhibitor HoPan (Figure 1A, [14]) resulted in decreased acetylation of the same proteins, an effect that was also reversed by pantethine (Figure 1D). HoPan treatment results in a block in CoA production in isolated mouse liver [14] and in decreased CoA levels in S2 cells (Figure S1). To further prove that observed acetylation defects are indeed sensitive and responsive to CoA levels, we supplemented the growth medium of dPANK/Fbl-depleted cells with increasing concentrations of CoA. Addition of CoA restored acetylation levels of the indicated proteins in a dose dependent manner (Figure 1E, for quantification see Figure 1F).

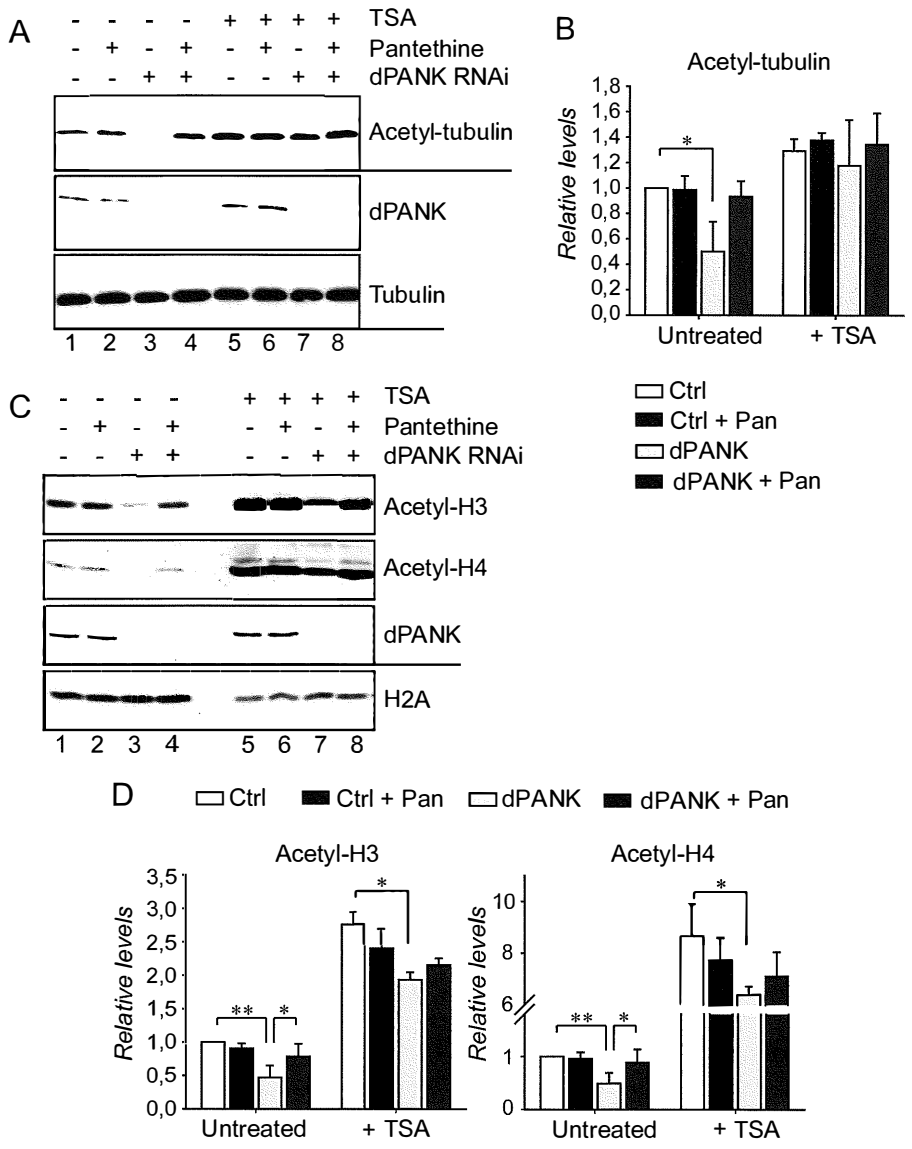
Our results demonstrate that the acetylation levels of at least two proteins are affected under circumstances of reduced CoA. To test whether the effect of dPANK/Fbl-depletion on protein acetylation of the specific proteins was not due to a general increased activity of deacetylases, we measured HDAC activity in the cell extracts. No significant differences in deacetylation rates between control and dPANK/Fbl depleted cells were observed (Figure S2). Next we tested whether acetylation of the specific proteins could be restored by inhibition of HDACs. Treatment with the HDAC inhibitor Trichostatin A (TSA) resulted in increased acetylation levels of the affected proteins, although acetylation levels in dPANK/Fbl depleted cells remained lower as compared to control cells (Figure 1B, right panel, compare lane 5 and lane 7). Together, these data

indicate that reduced acetylation levels of specific proteins in the dPANK/Fbl-depleted background are not caused by increased activity of HDACs, and are most likely the result of decreased levels of CoA. Independent of the reduced CoA levels, the reduced acetylation levels of these specific proteins can be partly restored by inhibiting HDAC activities. This further suggests that normal levels of CoA are required in addition to the balance between HATs and HDACs activities to maintain the proper status of acetylated lysine residues of specific proteins. The acetyl-lysine antibody used in this study possibly recognizes only a minor subset of acetylated proteins and most likely the indicated proteins are among the ones that are abundantly acetylated. In addition, acetylation levels of the indicated proteins, in contrast to the other proteins recognized by the acetyl-lysine antibody, respond most strongly to treatment with HDAC inhibitors and to impaired CoA biosynthesis, suggesting high acetylation/deacetylation dynamics of these specific proteins. In order to evaluate the complete spectrum of acetylated proteins affected by decreased CoA levels, more sensitive assays are required. Here we will focus on the proteins indicated in Figure 1B.

Decreased levels of CoA coincide with decreased acetylation of histones and tubulin

Next we identified the indicated proteins starting with the protein migrating at 55 kD. Previously it has been shown that tubulin is a protein that can be acetylated [15, 16] and its molecular weight matches with the protein indicated by the upper asterisk in Figure 1B. Western blot analysis using antibodies that recognize acetylated-tubulin confirmed that indeed in dPANK/Fbl depleted cells, levels of acetylated-tubulin, but not the total levels of tubulin are decreased (Figure 2A, compare lane 1 with lane 3, see Figure 2B for quantification). Acetylation levels of tubulin in dPANK/Fbl depleted cells are restored by addition of pantethine and by addition of the HDAC inhibitor TSA, (Figure 2A and 2B) confirming further the results of Figure 1B-C. Altogether these data demonstrate that a decrease in CoA levels coincides with decreased levels of acetylated-tubulin.

Next we aimed to investigate the identity of the lower bands (indicated by the lower asterisk in Figure 1B). The migration pattern of the low molecular weight proteins showing decreased acetylation in CoA-deficient S2 cells correlates well with molecular weights of histone proteins. Histones are among the first and most extensively studied proteins known to be abundantly acetylated [17]. We used antibodies that specifically recognize acetylated lysines of histone 3 and 4 to investigate whether the lower candidate bands represent acetylated histones. Indeed, the acetylation of histone 3 and histone 4 is 60% decreased in dPANK/Fbl-depleted cells (Figure 2C, compare lane 1 and lane 3, for quantification see Figure 2D), which is in agreement with the results presented in Figure 1. Decreased levels of acetylated histones were rescued by addition of pantethine and TSA (Figure 2C-D), demonstrating that CoA levels (and not levels of pantothenate kinase per se) influence the acetylation status of histones.



Taken together, using our *in vitro* model we have identified a tight link between cellular CoA levels and acetylation of proteins involved in the architecture of the cytoskeleton (via acetyl-tubulin) and in the integrity of the epigenome (via acetylation of histone tails). Further we aimed to identify the cellular and physiological importance of these specific acetylation defects induced by impairment of pantothenate kinase activity.

◀ Figure 2. Levels of acetylated tubulin and histones are decreased in *dPANK/fbl* depleted cells.

A, Cell extracts of control cells and dPANK/Fbl-depleted cells (by RNAi) were analyzed by Western blot using antibodies specifically recognizing acetylated-tubulin. Efficiency of RNAi was determined by using a *dPANK/fbl* antibody and tubulin was used as a loading control. Control cells and dPANK/Fbl depleted cells were left untreated, treated with pantethine, with TSA or with TSA and pantethine. B, Quantification of the relative levels of tubulin acetylation under the conditions presented in A compared to control cells C, Cell extracts of control cells and dPANK/Fbl-depleted cells were analyzed using Western blot to determine acetylation levels of specific histones. Specific antibodies were used to detect levels of acetylated histone 3 and acetylated histone 4. Control and dPANK/Fbl-depleted cells were left untreated or were treated with pantethine , with TSA or with TSA and pantethine. The efficiency of the RNAi treament was investigated by the use of an antibody against dPANK/Fbl. H2A was used as a loading control. D, Quantification of the relative levels of histone acetylation under the conditions presented in C compared to control cells.

Decreased tubulin acetylation coincides with impaired touch response in *C. elegans* pantothenate kinase mutants

Although in *Drosophila*, similar to other species, tubulin undergoes acetylation at the lysine 40 residue, the physiological importance of this modification in flies or *Drosophila* cell lines has not been demonstrated. On the contrary, in Caenorhabditis *elegans* reduced acetylation of tubulin has been recently linked with an impaired touch response [18]. To investigate whether the link between CoA metabolism and tubulin acetylation is evolutionarily conserved, we first tested if impaired function of pantothenate kinase coincides with reduced acetylation levels of tubulin in *C. elegans* as well, using a *C. elegans* mutant carrying a deletion of 773 bp within the *pnk-1* gene, an ortholog of human PANK2 (Figure S3, supplementary information and [9]). As revealed by Western blot analysis, the *pnk-1* mutant animals showed decreased levels of acetylated tubulin, which could be rescued, similarly to the *Drosophila* model, with the addition of pantethine to the food (Figure 3A). In agreement with the reported link between tubulin acetylation and the function of touch receptor neurons in *C. elegans*, *pnk-1* mutant worms showed a decreased touch response, which was also rescued by pantethine feeding (Figure 3B). Together these data strongly indicate a conserved link between CoA metabolism and tubulin acetylation.

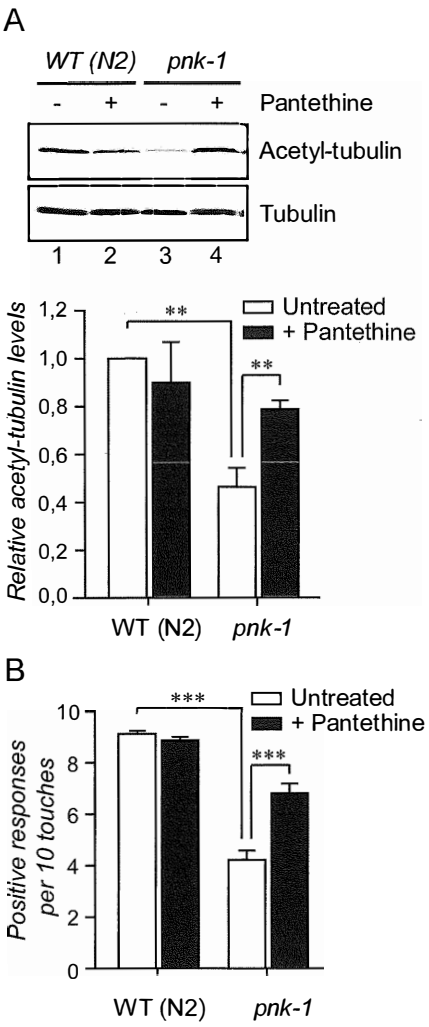


Figure 3. Decreased levels of acetylated tubulin in *C. elegans* pnk-1 mutants coincide with an abnormal touch response.

A, Extracts of staged L4 + 2 wild type (WT) and *pnk-1* mutant (pnk-1) animals were analyzed by Western blot using antibodies specifically recognizing acetylated-tubulin. Tubulin was used as a loading control. B, Touch responses were scored as previously described [18] in wild type animals and in *pnk-1* mutants under control conditions and after addition of pantethine to the medium. Error bars indicate SEM.

Decreased levels of CoA are associated with an impaired DNA damage response in a *Drosophila* model for PKAN

Next we investigated whether defects in histone acetylation correlate with specific phenotypes observed in the *Drosophila* PKAN model. *Drosophila* mutants that carry a mutation in genes coding for various enzymes required for the *de novo* synthesis of CoA (dPANK/Fbl, dPPCS, dPPAT-DCPK) demonstrate increased sensitivity to DNA damaging agents [12]. It is currently unknown why mutants that suffer from decreased levels of CoA are hypersensitive to DNA damage. Changes in histone acetylation are tightly linked with a competent DNA damage response [19] and increased acetylation of specific histone tails has already been reported in yeast, flies and humans after induction of DNA damage [20-23]. We investigated histone acetylation in response to induced DNA damage under circumstances of reduced levels of CoA. Hereto, control cells and dPANK/Fbl-depleted cells were irradiated to induce DNA double strand breaks and the dynamics of histone acetylation were investigated at various time points (Figure 4A). In control cells, a rapid histone acetylation was observed within 15 minutes after irradiation. The histone acetylation levels returned to those of control conditions during recovery of the cells. Increased histone acetylation was observed for various lysine residues (Lys5 of histone 2, Lys9 of histone 3 and Lys16 of histone 4), indicating the presence of a general histone acetylation induction in response to impaired DNA integrity. In dPANK/Fbl-depleted cells, this response was impaired, for all the lysine residues tested (Figure 4A). dPANK/Fbl-depleted cells showed a markedly smaller increase in histone acetylation (as for H4K16-ac), or a smaller increase combined with delayed increase in histone acetylation (as for H3-ac and H2K5-ac).

Next we investigated whether dPANK/Fbl depleted cells, like *Drosophila* CoA mutants are more sensitive to irradiation. Hereto, dPANK/Fbl-depleted and control cells were irradiated with various doses of ionizing radiation, and 6 days after exposure the number of surviving cells was determined. dPANK/Fbl-depleted cells showed a reduced survival as compared to control cells (Figure 4B). To further investigate the influence of decreased histone acetylation on radiation sensitivity, HDAC inhibitors were used and their influence on the survival of dPANK/Fbl deficient cells was tested. HDAC inhibitors (TSA or sodium butyrate (NaBut) were given 4 hours prior to ionizing radiation and the increase in acetylation was confirmed by measuring levels of acetylated histone 3 (Figure S4A). Pretreatment with HDAC inhibitors did not affect survival of the control RNAi cells. However, dPANK/Fbl-depleted cells showed a significantly higher survival when pretreated with HDAC inhibitors as compared to untreated dPANK/Fbl-depleted cells. Increasing histone acetylation by these means improved the cell survival by approximately 20 % (Figure 4B).

Together these data underscore the importance of global histone acetylation in DNA damage responses, as is in agreement with previous results by others [19-23]. Further, our data indicate that hypersensitivity to genotoxic stress of CoA depleted cells may, at least partially, be explained by the impaired acetylation levels of chromatin components.

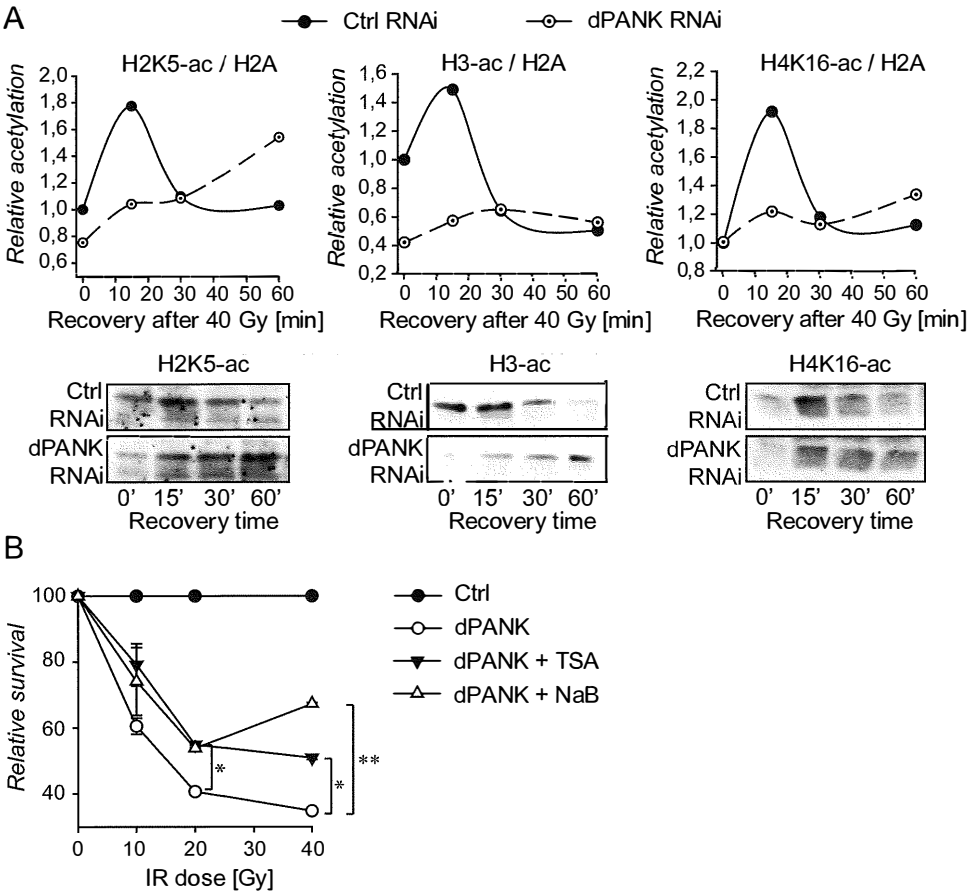


Figure 4. Decreased levels of acetylated histones and tubulin are associated with increased sensitivity to irradiation of dPANK/Fbl-depleted cells.

A, Control cells and dPANK/Fbl-depleted cells were irradiated (40 Gy) and acetylation levels of specific histone tails were determined after various time points. Levels of H3Ac, H4K16Ac and H2K5Ac were determined using specific antibodies for each histone or histone tail. The blots and the quantifications are representative of three independent experiments. B, Relative cell survival of control cells and dPANK/Fbl-depleted cells was measured after various doses of irradiation (10 Gy, 20 Gy, 40 Gy). Cell survival was also determined after treating the cells with HDAC inhibitors (TSA or NaB) (See Figure S4A for acetylation levels). Survival of untreated control cells was set to 100% for every dose of irradiation.

Decreased levels of CoA is associated with impaired locomotor function in a *Drosophila* model for PKAN

The *Drosophila* model for PKAN is further characterized by a decreased survival rate, neurodegeneration and by impaired locomotor function [11-13, 24]. The impaired locomotor function is most likely (at least partly) caused by the neurodegeneration. We next investigated whether these phenotypes also correlate with decreased acetylation levels. Especially acetylation of tubulin and histones as we report here for dPANK/Fbl depleted cells have previously been shown by others to be associated with neurodegeneration and with abnormal neuronal functioning [15, 18, 25-30]. In the experiments as described above *Drosophila* S2 cultured cells were used and first we tested whether in *Drosophila dPANK/fbl* mutant whole organisms (*Drosophila* PKAN model) levels of acetylated tubulin and histones were also decreased. Western blot analysis using extracts of third instar larvae indeed demonstrated that levels of acetylated tubulin and histones were decreased in *dPANK/fbl* homozygous larvae as compared to wild type larvae (Figure 5A, compare lane 1 and lane 3, for quantification see Figure 5B). Homozygous *dPANK/fbl* flies show a reduced eclosion rate, evidenced by the relative low number of homozygous adults compared to heterozygous adults (the ratio heterozygous:homozygous adult survivors is 16, whereas based on genetic inheritance this is expected to be 2). First we investigated whether addition of various HDAC inhibitors (valproic acid (VPA), sodium phenylbutyrate (PBA) or TSA) to the larval food increased the eclosion rate of homozygous *dPANK/fbl* flies. VPA and PBA did not result in a significant rescue (Figure S5) however, TSA addition increased the survival rate of the homozygous mutant progeny in a concentration dependent manner (Figure 5D). VPA and PBA could only be used in relatively low concentrations, because the concentrations commonly used for an efficient HDAC inhibition (above 1 mM) induced lethality when fed during larval development. TSA, on the other hand, is less toxic. Moreover, TSA is a potent and broad spectrum inhibitor acting on all *Drosophila* HDACs [31, 32]. The most effective concentration of TSA (0,2 μM) was used for further studies and we demonstrated that this induces a partial restoration of the decreased levels of acetylated tubulin and histones in *dPANK/fbl* mutant larvae (Figure 5A-B). This coincided with an increase in locomotor function assessed by larval crawling as a read-out assay (Figure 5C).

These data suggest a correlation between tubulin- and histone-acetylation levels and *dPANK/fbl* mutant phenotypes. These data are however not conclusive as to whether restoration of only these specific proteins is sufficient for improvement of locomotor function and survival, because acetylation levels of other proteins may also restore upon TSA feeding. Nonetheless, the tight correlation between CoA levels, acetylation of tubulins and histones and the specific phenotypes in dPANK/Fbl depleted cells and flies suggests that an altered status of acetylation of specific proteins may explain the pleiotropic mutant phenotype.

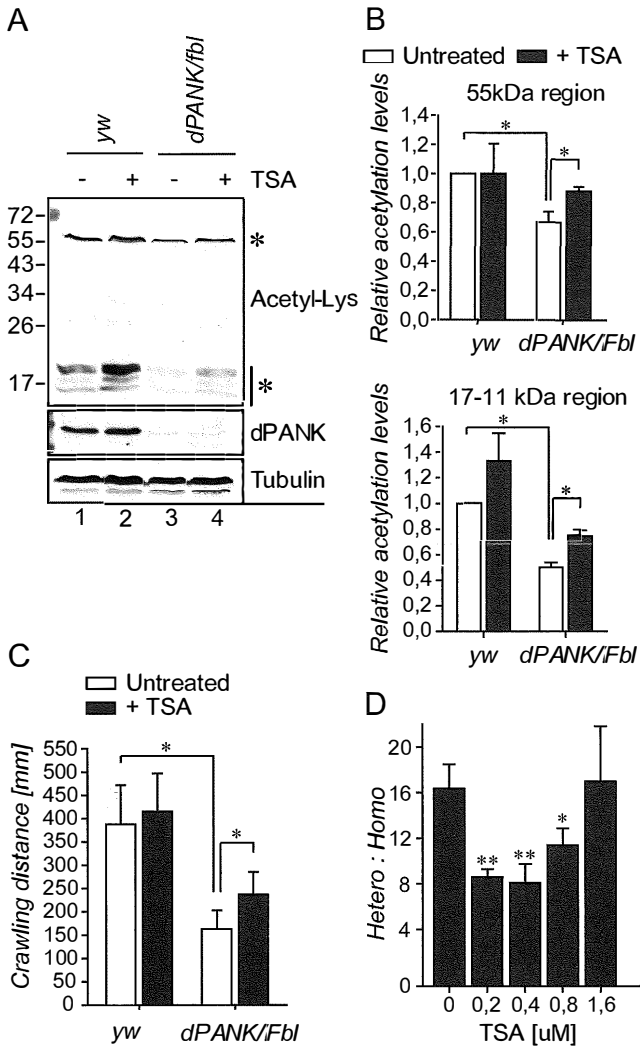


Figure 5. Decreased levels of acetylated histones and tubulin are associated with decreased survival and decreased locomotor function of *dPANK/fbl* mutant larvae.

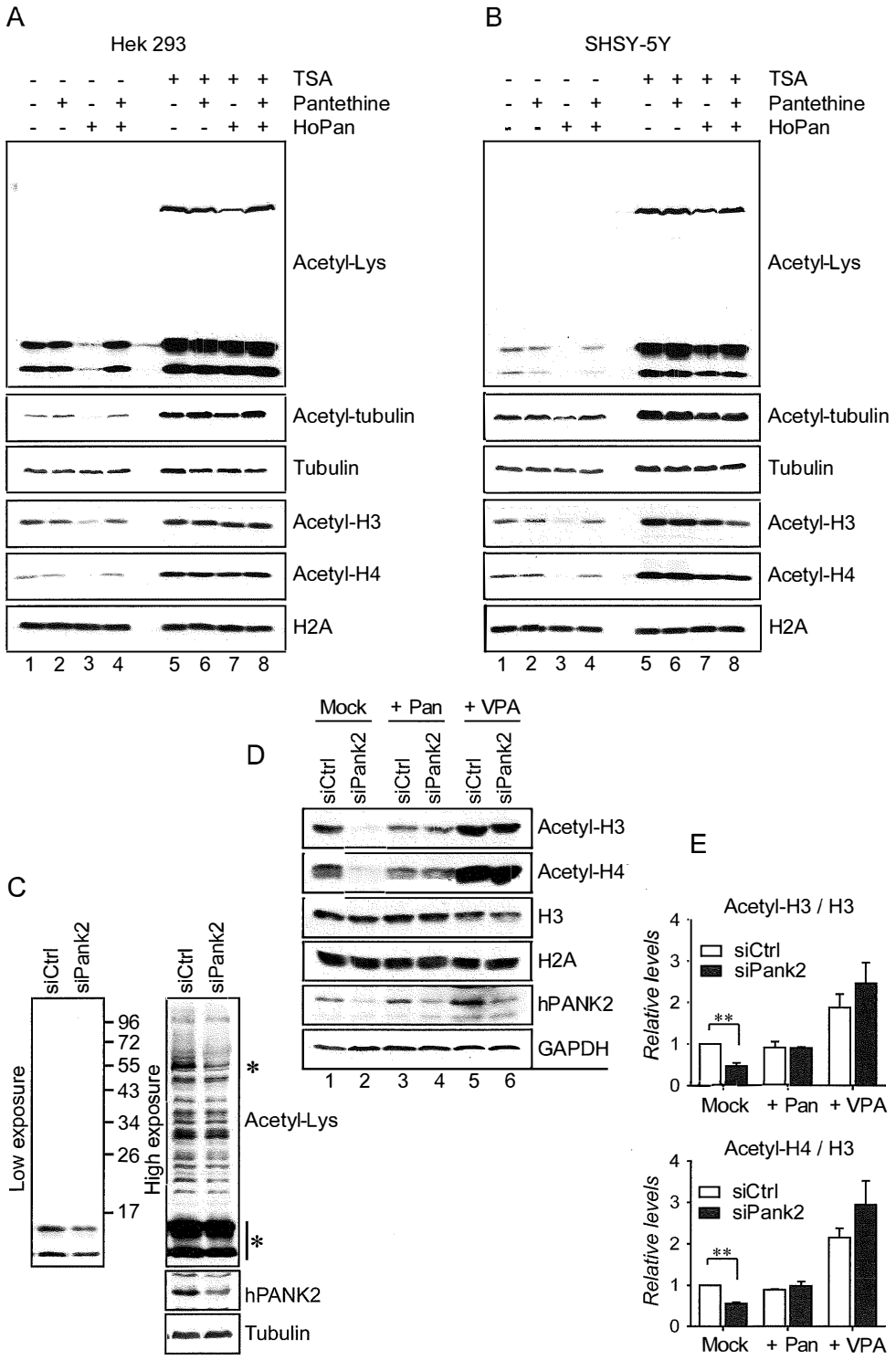
A, Extracts of wildtype and *dPANK/fbl* homozygous third instar larvae were analyzed for their levels of acetylated proteins using acetyl-Lys antibody. Tubulin was used as a loading control and the dPANK antibody was used to demonstrate the reduced expression of dPANK/Fbl in the mutant larvae. Addition of 0,2 µM TSA to the larval food resulted in increased levels of acetylated histones and tubulin. B, Quantification of the relative intensity of the 55 kDa band (corresponding to acetyl-tubulin) and <17 kDa bands (corresponding to acetyl-histones) in larvae extracts. C, Ability of larvae to crawl a certain distance in 9 minutes was measured as previously described [13]. Larval crawling assay was performed in wildtype larvae and in *dPANK/fbl* homozygous mutant larvae untreated or fed with the HDAC inhibitor TSA (0,2 µM). D, *dPANK/fbl*/TM3 males and females were crossed and various concentrations of TSA were added to the food. The number of homozygous (*dPANK/fbl*/*dPANK/fbl*) versus heterozygous *dPANK/fbl*/TM3 adults which eclosed was counted.

Impairment of pantothenate kinase function correlates with decreased acetylation of tubulin and histones in various mammalian cell models for PKAN

Finally, we aimed to test whether inhibition or downregulation of pantothenate kinase in a human cell lines also results in decreased levels of acetylated tubulin and acetylated histones. Firstly, human pantothenate kinase activity was inhibited using HoPan in HEK293 cells (Figure 6A). Western blot analysis using the acetyl-lysin, the acetyl-tubulin, the acetyl-H3 and the acetyl-H4 antibodies demonstrated that levels of acetylated tubulin and levels of acetylated histones (Figure 6A) were decreased. These effects were rescued by addition of pantethine or TSA to the medium (Figure 6A). In light of the above data, it is highly relevant to investigate levels of histone and tubulin acetylation in material derived from PKAN patients. We investigated acetylation levels in available patient-derived lymphoblasts. In these cells no significant difference in histone acetylation could be observed (Figure S6). However, it should be noted that lymphoblast cells of patients do not show any phenotype and therefore it will be of higher relevance to investigate protein acetylation levels in tissues (when available in the future) that are affected in PKAN patients, such as the globus pallidus [33].

In order to test whether neuronal cells show the same response to impaired pantothenate kinase function, SHSY-5Y cells, (a human derived neuroblastoma cell line [34]) were used and HoPan treatment induced a decrease in tubulin and histone acetylation, an effect reverted by pantethine or TSA treatment (Figure 6B).

Finally, we aimed to test whether downregulation of specifically PANK2 (the causative gene of the neurodegenerative disease PKAN) in a human cell line also resulted in decreased levels of acetylated tubulin and acetylated histones. Human PANK2 was down-regulated using siRNAs in HEK293 cells (Figure 6C). Western blot analysis using the acetyl-lysin antibody demonstrated that levels of acetylated tubulin (Figure 6C, high exposure) and levels of acetylated histones (Figure 6C low exposure) were decreased. These results were further confirmed using specific antibodies against acetyl H3 and acetyl H4 and hPANK2 depletion resulted in a 50% decrease in histone 3 as well as histone 4 acetylation as compared to cells treated with control siRNA (Figure 6D, for quantification see Figure 6E). Addition of pantethine to the cell culture medium increased the acetylation in siPANK2 treated cells to the level indistinguishable from that of control cells (Figure 6D). These results demonstrate that in human cells, impaired *de novo* biosynthesis of CoA is also associated with decreased levels of acetylation of a specific set of proteins. Additionally, we tested valproic acid (VPA) for its potential to increase acetylation in PANK2-depleted background. VPA is an HDAC inhibitor of a high interest for mammalian systems because it is able to cross the blood-brain-barrier and has been proposed as a possible treatment for neurodegeneration (reviewed in [30]). Treatment with VPA increased acetylation levels of histones in hPANK2-depleted cells (Figure 6D and E).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Hek 293
TSA
Pantethine
HoPan
Acetyl-Lys
Acetyl-tubulin
Tubulin
Acetyl-H3
Acetyl-H4
H2A
1 2 3 4 5 6 7 8
B
SHSY-5Y
TSA
Pantethine
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1 2 3 4 5 6 7 8
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+ Pan
+ VPA
Acetyl-H4 / H3
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◀ Figure 6. Impairment of human PANK activity *in vitro* results in decreased levels of tubulin and histone acetylation, an effect reverted by pantethine and by HDAC inhibitors.

A, HEK293 cells were left untreated or were treated with HoPan. Control and HoPan treated cells were co-treated with pantethine or TSA. Acetyl-lysine, acetyl-tubulin, acetyl-H3 and acetyl-H4 antibodies were used to analyse acetylation levels of tubulin and histones. Histone H2A and tubulin were used as loading controls. B, As in A but human SHSY-5Y cells were used. C, HEK293 cells transfected with a control siRNA or with an siRNA against human PANK2 were analyzed for the levels of acetylated proteins. Acetyl-lysine antibody was used. Low exposure (left panel) was shown to determine levels of histone acetylation (lowest asterisk), high exposure (right panel) was shown to determine levels of acetyl-tubulin (highest asterisk). Efficiency of RNAi was confirmed using an antibody specifically recognizing human PANK2. Tubulin was used as a loading control. D, Control cells and human PANK2 depleted cells were investigated for their levels of acetyl H3 and acetyl H4. Cells were additionally left untreated or were treated with pantethine or valproic acid. Specific antibodies were used to determine levels of histone H3 and histone H4. The efficiency of human PANK2 RNAi was determined by a specific human PANK2 antibody. H3 and GAPDH were used as loading controls. E, Quantification of the relative levels of acetylated histone 3 and acetylated histone 4 compared to control cells.

Discussion

In this study we show that in addition to H/KATs and HDACs, which are the key proteins controlling protein acetylation, levels of the metabolite CoA affect the status of protein acetylation as well. We demonstrate that under conditions of reduced levels of CoA, there is no compensatory mechanism able to maintain normal histone and tubulin acetylation. Numerous reports exist about the role of CoA in metabolic processes (reviewed in [8]), however an influence of levels of this cofactor on protein acetylation as we describe here has never been reported. Recent results of other studies are in line with our observations. It has been demonstrated that enzymes required for the synthesis of acetyl-CoA from acetate or citrate also influence the acetylation status of histones [5-7]. Although neither CoA nor acetyl-CoA levels were directly measured in these studies, these results are in agreement with our observations and with the model presented in Figure 7. Our results reveal that levels of tubulin and histone acetylation are decreased but still detectable under conditions of CoA reduction. In addition, acetylation levels of other proteins recognized by the acetyl-lysin antibody seem to be unaffected. It will be of interest to investigate how much residual CoA is required to maintain acetylation levels of specific proteins. Until now no literature exists addressing these issues. Previously we demonstrated that in adult *dPANK/fbl* (hypomorph) mutant flies levels of CoA are undetectable with the method used [13], however we cannot conclude that CoA levels are actually zero. In dPANK/Fbl RNAi depleted *Drosophila* S2 cells, levels of CoA decrease to 30% [13] and after HoPan treatment CoA levels drop to 50% (this manuscript). Under all these circumstances, acetylation of tubulin and histones is still detectable but clearly decreased. Most likely under these conditions there is a residual CoA and acetyl-CoA pool left, sufficient for the detected protein acetylation or alternatively there exist an additional source other than acetyl-CoA for protein acetylation. It will be of interest to investigate in a more detailed way, how levels of CoA in specific subcellular compartments influence the acetylation of specific proteins over time and whether this is tissue specific and how this affects specific cellular processes.

The importance of normal CoA metabolism is underscored by the fact that PKAN patients who carry a mutation in the pantothenate kinase 2 gene suffer from an early onset and a severe form of neurodegeneration [9]. The pathophysiology of this disease is not understood. Recently, we showed using a *Drosophila* PKAN model that levels of CoA are severely reduced when pantothenate kinase is affected [13]. Here we show that reduced levels of CoA coincide with reduced acetylation levels of histones and tubulin and it is of interest that these "marker" proteins are associated with neurodegenerative conditions reviewed in [28, 29]. Our observations suggest that at least part of the impaired locomotor abnormalities of the *Drosophila* PKAN model can be explained by decreased acetylation levels of specific proteins and this may also explain the complex pathophysiology of

PKAN patients. Although the above mentioned studies and our results together demonstrate a strong link between histone and tubulin acetylation and neurodegeneration, it should be stressed that neither the published studies nor our data exclude that acetylation levels of other proteins may also explain part of the observed effects.

Another feature that correlates with decreased levels of CoA and impaired histone- and tubulin acetylation in *Drosophila dPANK/fbl* mutants and dPANK/Fbl-depleted cells is their increased sensitivity to DNA damaging agents. This correlation is in line with other reports, suggesting that normal acetylation of histones is required for a functional DNA damage response [19]. Therefore, we propose that the DNA damage sensitive phenotype of the *Drosophila* PKAN model may be explained by decreased acetylation levels of a specific set of proteins. It is yet unclear whether or not the presence of DNA damage as a result of an impaired DNA damage response is somehow linked to neurodegeneration or impaired locomotor function, however, previously we demonstrated that inducing DNA damage in wildtype fruitflies results in reduced climbing activities [12] and there are multiple examples of neurodegenerative diseases associated with an impaired DNA damage response (reviewed in [35]).

Our data demonstrate that defects in panothenate kinase function in various cells and organisms are associated with a decrease in acetylated tubulin. This is of high interest because there is extensive literature in which abnormal tubulin acetylation is linked to impaired neuronal functioning. Amongst others, acetylated tubulin is associated with stable tubulin filaments and defects in tubulin acetylation are associated with abnormal transport in neuronal cells [25], abnormal branching of projection neurons [26], neuromuscular defects [15] and impaired function of touch receptor neurons [18] (for a recent review see Perdiz et al [36]).

Although it remains to be proven it is attractive to speculate that the neurological defects observed in PKAN affected individuals may be partly explained by abnormal tubulin acetylation.

All together our results indicate a conserved link between CoA metabolism and protein acetylation and when disturbed this coincides with a pleiotropic phenotype as observed in the *Drosophila* model for PKAN[9, 11-13]. These results provide a novel explanation for how the disturbed synthesis of CoA as in PKAN may lead to neurodegeneration. We furthermore show that the *Drosophila* PKAN phenotype is partly rescued by adding specific compounds to the food that either replenish CoA levels (pantethine) or restore protein acetylation levels (TSA). This knowledge can be of use to develop possible future therapies for PKAN.

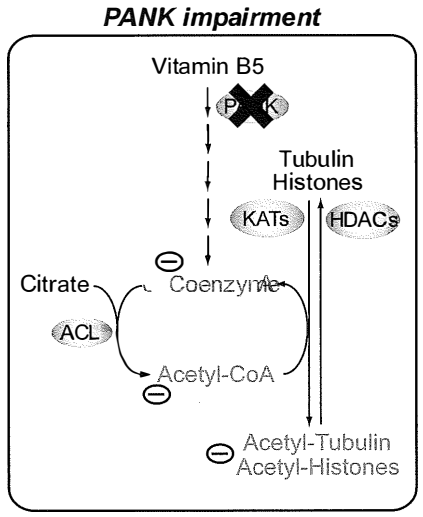

Figure 7. CoA metabolism and protein acetylation are tightly linked.

Under wildtype conditions, CoA is synthesized *de novo* from Vitamin B5 at normal levels. CoA is incorporated in acetyl-CoA and the latter serves as the acetyl source for various protein acetylation reactions. A tight interplay between KATs and HDACs determine the acetylation levels of specific proteins resulting in normal homeostasis of cells and tissues. In cells and organisms suffering from impaired function of pantothenate kinase, CoA *de novo* biosynthesis is disturbed, and levels of CoA are decreased. Reduced levels of CoA result in decreased levels of acetyl-CoA and reduced acetylation of tubulin and histones. The latter is associated with decreased survival, impaired locomotor function and an impaired DNA damage response. We suggest that decreased acetylation levels of specific proteins may explain part of the pleiotropic phenotype of the *Drosophila* PKAN model and possibly part of the pathogenesis of PKAN.

Materials and Methods

Cell culture and RNAi - *Drosophila* Schneider's S2 cells were cultured and subjected to RNAi treatment as described previously[13]. HEK293 and SHSY-5Y cells were cultured according to the standard protocols in dMEM supplemented with 10 % FCS and Penicilin/Streptomycin. For RNAi experiments 50 nM PANK2 siGENOM SMARTpool (Dharmacon) or non-silencing control siRNA (Dharmacon) were transfected with Lipofectamine (Invitrogene) for 6 hours in serum free medium. Transfection medium was then removed and cells were cultured for 48 hr in complete culture medium. Cells were subcultured and transfected again according to the same protocol. 6 hours after second transfection complete medium with or without 0,1 mM pantethine was added. Samples were collected 48 hours after the second transfection. For HoPan treatment HEK293 and SHSY-5Y cells were cultures in custom made vitamin B5 deficient dMEM (Thermo Scientific) supplemented with dialyzed FCS (Thermo Scientific). This medium did not affect normal growth of the cell lines. For all the cell lines studied, pantethine was used in the culture medium at the final concentration of 0,1 mM. HEK

293 and SHSY-5Y cells were treated with HoPan and/or pantethine for 4 and 6 days respectively.

Inhibitors - Pantothenate kinase inhibitor, HoPan, was previously described [14] and was used in the cell culture medium at a final concentration of 0,5 mM. HDAC inhibitors: Trichostatin A (TSA), sodium butyrate and valproic acid (VPA) were purchased from Sigma. *Drosophila* S2 cells were treated with 0,5 μM TSA for 24 hours, unless mentioned otherwise. Sodium butyrate was used in a final concentration of 5 mM for 5 hours. HEK293 and SHSY-5Y cells were treated with 0,25 μM TSA for 7 hours or with 2mM VPA for 48hr.

Immunoblotting - Immunoblotting was performed as previously described [13]. The following antibodies were used: anti-dPANK/Fbl (previously described [12]), anti-acetyl-lysine (Cell Signaling, #9441), anti-alpha-tubulin (Sigma), anti-acetyl-tubulin (Sigma, clone 6-11B-1), anti-acetyl-H3 (Active Motif, #39139), anti-acetyl-H4 (Millipore, #06-598), anti-H2A (Abcam, #ab13923), anti-acetyl-H4K16 (Active Motif), anti-acetyl-H2K5 (Active Motif), anti-H3 (Millipore), anti-hPANK2 (a gift from J. Gitschier, UCSF) anti-GAPDH (Fitzgerald Industries). HRP-conjugated secondary antibodies were from Amersham. Western blots from at least 3 independent experiments were quantified using Adobe Photoshop SC3.

S2 cells - radiation survival assay - RNAi experiment was performed to knockdown dPANK protein. After 4 days of RNAi cells were subcultured to an equal density and left untreated or treated with HDAC inhibitors. After 4 hours cells were washed twice with PBS, resuspended in complete culture medium and plated in the density of 0,4 x 10^6/ml in 36mm dishes. Cells were irradiated with Cesium-137 source IBL 637 irradiator (CIS Bio-Internationl). Six days after exposure to IR viable cells were counted with Trypan Blue exclusion test.

Drosophila maintenance and physiological assays - Hypomorphic *dPANK/fbl*^{1} strain was used [11, 12], y1w^{1118} were used as a wild type control (Bloomgton Stock Centre). TSA feeding experiments: fly food was prepared by addition of varying concentration of TSA or DMSO (as control) to the standard food, equal numbers of flies (12 females, 4 males) were placed in vials and allowed to lay eggs for 48 hours. Late third instar wild type and homozygous *dPANK/fbl*^{1} larvae were collect for analysis. Larval crawling assays were performed as described before [13]. To measure the eclosion rates of homozygous *dPANK/fbl*^{1} on TSA supplemented or control food, the ratio between heterozygous and homozygous adult survivors in the F1 generation was determined.

C. elegans - *C. elegans* strains were maintained at 20°C, according to the standard protocols [37]. N2 was used as a wild type strain and the PANK deletion mutant, VC927 *pnk-1* (ok1435)I/hT2[bli-4(e937) let-? (q782)qIs48](I;III), was obtained from the Caenorhabditis Genetics Center. Touch response assays were performed by blind scoring. L4 larvae were isolated and placed on control NGM agar plates or NGM agar plates supplemented with 0,8 mg/ml pantethine and seeded with E. coli OP50. After 48 hours each animal was touched 10 times with an eyelash, altering between the anterior and posterior part of the body. Positive response as movement away behavior from the stimulation was scored for 60 worms per condition. For western blotting 40 - 50 synchronized worms were collected in 25 µl of M9 buffer, frozen in liquid nitrogen and stored in - 80°C. 25 µl of 2 X Laemmli sample buffer (without Bromophenol Blue) was added and samples were homogenized by sonication. Protein concentration was measured with DC Protein Assay (Bio-RAD), sample volumes were adjusted with 1 x Laemmli sample buffer (with Bromophenol Blue) and equal amounts of protein were loaded on SDS-Page gels.

Statistics - Statistical data significance was estimated using the Student's t-test (2-tailed and where appropriate with equal or unequal variance). Plotted values represent averages of at least 3 independent experiments and error bars represent standard deviation. P-values below 0,05 were consider significant, where $p < 0,05$ was indicated with *, $p < 0,005$ with ** and $p < 0,001$ with ***.

Author contributions

KS, BS, LX, AR, EAAN, SJ, SH and OCMS designed the experiments. KS, BS, AR, LX, LS and JJ performed the experiments. KS, BS, EAAN, SJ, SH and OCMS wrote the paper.

Acknowledgements

We thank Harm Kampinga, Floris Bosveld and Sarah Pringle for critical reading of the manuscript and Karen Thijssen for technical assistance. The work was supported by a VIDI and VICI (Dutch organization for scientific research) grant to O.S and a GUIDE (Groningen Graduate school) grant to K.S.

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Supplementary Figures

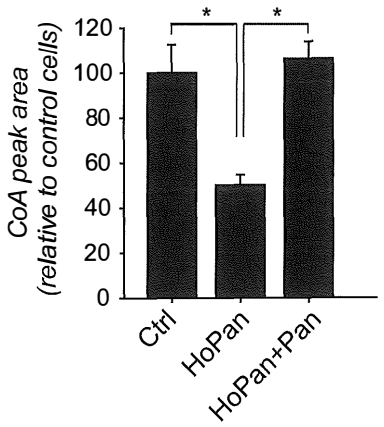


Figure S1. CoA levels are decreased in HOPAN treated S2 cells.

S2 cells were left untreated, treated with HOPAN (0.5 mM) or treated with HOPAN and pantethine (0.1 mM) and CoA levels were measured (see Supp Methods) after for 48 hours.

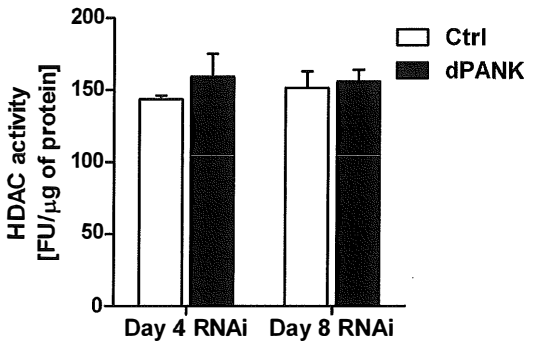


Figure S2. dPANK RNAi in *Drosophila* S2 cells has no effect on HDAC activity.

HDAC activity was measured (see Supp. Methods) in control cells and in dPANK/Fbl depleted cells after 4 and 8 days of RNAi treatment.

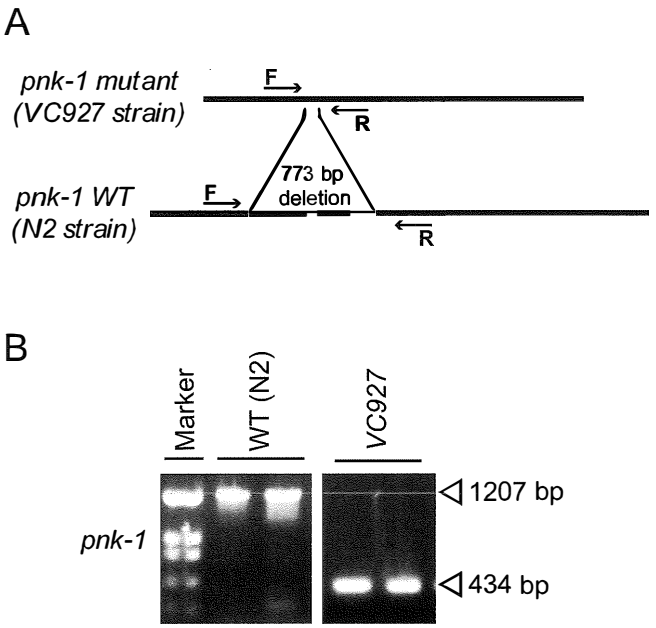


Figure S3. *C. elegans* mutant strain VC927 carries a deletion in pantothenate kinase gene *pnk-1*.

A, Schematic representation of the 773 bp deletion region in *pnk-1* gene present in the *C. elegans* VC927 strain. Arrows indicate the position of primers (forward - F and reverse - R) used to confirm the presence of the deletion. B, Single worm PCR amplification of a fragment of *C. elegans pnk-1* gene was performed to confirm the 773 bp deletion in VC927 strain used in this study. N2 worms were used as a wild type control. See supplementary material and methods for the details on the PCR reaction and primer sequences

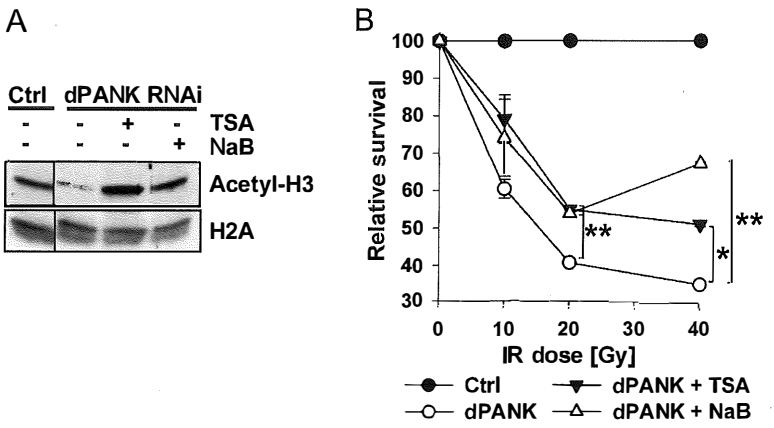


Figure S4. Decreased levels of acetylated histones coincide with radiation sensitivity of dPANK/Fbl-depleted S2 cells.

A, Cells were treated with HDAC inhibitors (TSA or NaB) 4 hours before the exposure to ionizing radiation. Acetylation of histone 3 was assayed with western blotting to demonstrate the effect of inhibitors. H2A was used as a loading control. B, Cell survival was determined after treating the control cells and dPANK/Fbl-depleted cells with HDAC inhibitors (TSA or NaB) followed by the exposure to increasing doses of ionizing radiation.

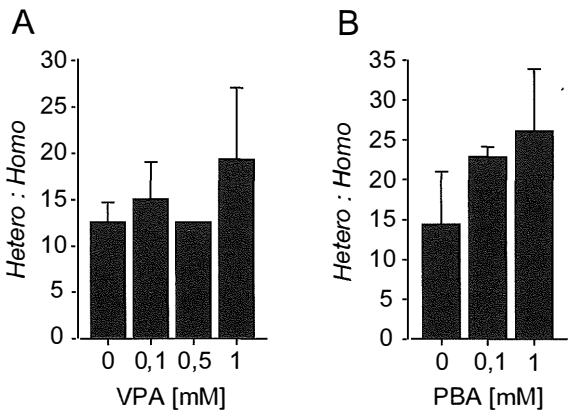


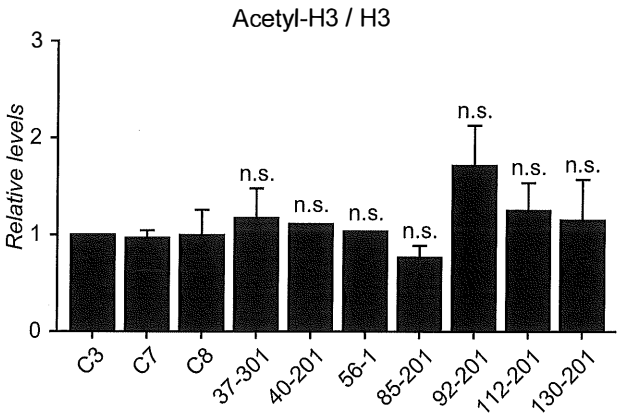
Figure S5. VPA or PBA feeding fails to improve the eclosion rates of dPANK/Fbl mutant flies.

dPANK/fbl mutant flies were raised on fly food supplemented with increasing doses of valproic acid (VPA) A, or sodium phenyl-butyrate (PBA) B, The eclosion rates were determined as described in Figure 5D and material and methods section. For both compounds, concentrations above 1 mM were toxic resulting with very low numbers of eclosed flies (regardless of a genotype).

A

Sample ID	*hPANK2* mutation
C3	None
C7	None
C8	None
37-301	206-228 del 243 del
40-201	502 C>T 1231 G>A
56-1	1231 G>A homozygous
85-201	526 C>T 1231 G>A
92-201	519 C>G 1231 G>A
112-201	1021 C>T 1231 G>A
130-201	993-996 del homozygous

B



C

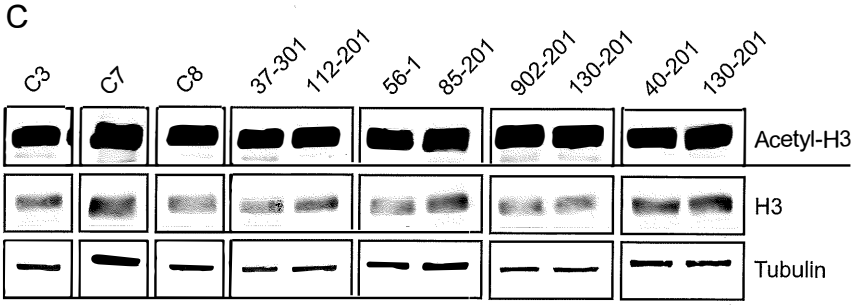


Figure S6. No significant changes in the levels of H3 acetylation can be observed in PKAN lymphoblasts derived from 7 patients.

Levels of acetylated histone 3 were determined by Western blott analysis using lymphoblasts derived from various PKAN patients and control healthy individuals. A, Three control lymphoblast lines (C3, C7 and C8) were analyzed in addition to seven patient-derived lines (37-301, 40-201, 56-1, 85-201, 92-201, 112-201, 130-201). A table indicates mutations present in the hPANK2 gene in each cell line tested. B, Quantification of the relative levels of acetyl-histone 3 in the lymphoblast lines described in A. Total H3 was used as a loading control and the value obtained for C3 control sample was set us 1. Error bars represent st.dev.; n.s. – not significant (vs. C3 control) C, Representative blots used for the analysis of acetyl-H3 levels as shown in B. As an additional loading control tubulin was visualized.

Supplementary Methods

Measurement of total CoA levels by HPLC - The levels of total CoA in *Drosophila* Schneider's S2 cells were measured after pre-column derivatization with ammonium 7-flurobenzo-2-oxa-1,3-doazole-4-sulfonate (SBD-F) using reverse phase HPLC-fluorescence detection. The pre-column derivatization procedure for both S2 cell samples and CoA standard dilutions was performed as previously described [1]. Briefly 10 million *Drosophila* Schneider's S2 cells were pelletized and washed with ice cold phosphate buffered saline. 100 uL of borate buffer (0.1M containing 1 mM EDTA disodium, pH 9.5) was added to the cell pellets and sonicated three times to lyse the cells. To the lysate 20uL Tributylphosphine (10% v/v in DMF) was added and allowed to react at room temperature for 10 min. Proteins were removed and 5 µL of ammonia (12.5%) was added to an aliquot of the resulting solution (50 µL) and derivatized with 45 µL solution of SBD-F (1mg/ml in borate buffer). The derivatized sample was then analyzed using High-Performance Liquid Chromatography (HPLC) in combination with a fluorescence detector with excitation at 385 nm and emission at 515 nm, using optimized chromatographic conditions (manuscript of complete chromatographic conditions and CoA derivatization/stability is under preparation and will be published elsewhere).

HDAC activity assay - S2 cells (5 million cells/sample) were pelleted, washed once with PBS and resuspended in 200 µl of cold HDAC Cell Lysis Buffer (Enzo Life Sciences). Lysates were incubated on ice for 15 min, followed by a brief homogenization by passing 5 times through a 26G needle. Homogenates were centrifugated at 10000 rpm, 4°C for 10 minutes and the supernatant was used for protein estimation and for the HDAC activity measurement. Protein content was determined with a DC Protein Assay (BioRad). HDAC activity was assayed with the fluorymetric HDAC Assay Kit (Sigma) according to manufacturer instructions. Fluorescence readings for each sample were averaged and corrected for the total protein levels.

C. elegans single worm PCR - Total DNA was obtained by a single worm lysis in 5 µl lysis buffer containing 10 µl/ml ProtK. Worms were lysed at 60°C for one hour, followed by 95°C for 15 minutes. 1 µL of the lysate was used for a standard PCR reaction. Primers were designed in front and behind the deletion part in the *pnk-1* gene: TTTGTCGCGAGTTCTTGTAAAGGCT (forward) and GAGCAGGTGTGAGCAGGCTTCC (reversed).

Measurement of the histone acetylation levels in PKAN patient lymphoblasts - Lymphoblasts from healthy and PKAN diagnosed individuals were derived from blood samples and cultured as previously described [2]. The exception to this was Control 8, which was obtained from the National Institute on Aging through the Coriell Cell Repositories (New Jersey, USA) and then cultured similarly to the other samples. Cells were counted, and equal numbers were spun down and harvested in Laemmli buffer, sonicated on ice, then boiled. Western blots were run using equal sample volumes and developed with secondary fluorescent antibodies. Blots were visualized/analyzed with a Licor Odyssey Infrared Imaging System and associated Odyssey software. Primary antibodies included Active Motif Histone H3 acetyl antibody (rabbit, 1:1500), Active Motif Histone H3, C-terminal antibody (rabbit, 1:25000), and monoclonal anti-γ-tubulin (mouse, 1:4000). Secondary antibodies included Invitrogen Alexa Fluor 680 anti-rabbit IgG (goat, 1:10000) and Rockland anti-mouse IgG Antibody IRDye800CW Conjugated (goat, 1:10000).

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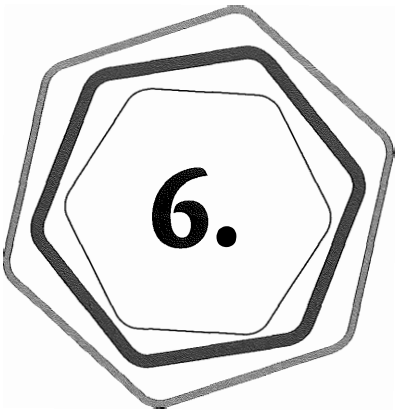

Hyperphosphorylation of actin severing protein cofilin in response to impaired Coenzyme A metabolism in *Drosophila* S2 cells and during neuronal differentiation *in vitro*

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Manuscript submitted

Abstract

Coenzyme A (CoA) is a pantothenic acid-derived metabolite essential for many fundamental cellular processes including energy, lipid and amino acid metabolism. Pantothenate kinase (PANK), which catalyses the first step in the conversion of pantothenic acid to CoA, has been associated with a rare neurodegenerative disorder PKAN. However, the consequences of impaired PANK activity are poorly understood. Here we use *Drosophila* and human neuronal cell cultures to show how PANK deficiency leads to abnormalities in F-actin organization. Cells with reduced PANK activity are characterized by abnormally high levels of phosphorylated cofilin, a conserved actin filament severing protein. The increased levels of phospho-cofilin coincide with morphological changes of PANK-deficient *Drosophila* S2 cells and human neuronal SHSY-5Y cells. The latter exhibit also markedly reduced ability to form neurites in culture – a process that is strongly dependent on actin remodeling. Our results reveal a novel and conserved link between a metabolic biosynthesis pathway and regulation of cellular actin dynamics.

Introduction

Regulation of actin dynamics is critical for cellular function. Cells respond to various external and internal stimuli by specific remodeling events of the actin cytoskeleton. Actin rearrangements are required for changes in cell shapes and thus actin dynamics are important for a variety of morphogenetic events such as cell divisions, cell migration, adhesion, neuritogenesis (sprouting of neurites) or exon pathfinding. At the same time, the regulation of these processes is tightly linked to the metabolic status of cells and organisms. Numerous data demonstrate the involvement of Rho family GTPases in the control of actin filament nucleation and disassembly [1, 2], however relatively little is known about whether and how actin cytoskeleton signaling is influenced by and tuned with the metabolic state of the cell.

Coenzyme A (CoA) is an central metabolite present in all living organisms [3]. CoA reacts with carboxyl groups giving rise to thioesters – CoA-activated acyl moieties. About 4 % of all known enzymes utilize CoA as a cofactor and CoA thioesters are essential for over 100 different reactions of the intermediary metabolism, such as the tricarboxylic acid cycle (TCA cycle), lipid synthesis and oxidation or the synthesis of some amino acids (reviewed in [3]). Hence, CoA occupies a central position in the regulation of the cellular metabolism. One evolutionary conserved pathway has been described leading to the *de novo* biosynthesis of CoA. The first step of this pathway is phosphorylation of pantothenic acid (vitamin B5) by pantothenate kinase (PANK). The pathway has gained renewed attention after the discovery that mutations in one of the four human PANK isoforms, PANK2, lead to a severe neurodegenerative disorder, Pantothenate Kinase-Associated Neurodegeneration (PKAN) [4]. Recently, we and others have established a *Drosophila melanogaster* model for PKAN [5-8] and we have shown that *Drosophila dPANK/fbl* mutant flies as well as downregulating dPANK/Fbl using an *in vitro* RNAi approach in *Drosophila* Schneider's S2 cells constitute suitable models for studying the consequences of CoA deficiency. The *dPANK/fbl* gene was initially identified in a screen for male sterility and *dPANK/fbl* mutants show cell division errors and cytokinesis defects with abnormal F-actin dynamics [5, 8]. Additionally, abnormal F-actin accumulation is observed in the ovaries of CoA deficient female flies, which are also sterile [9]. This correlation of CoA metabolism in flies with actin related processes suggests additional, yet not appreciated, influences of CoA levels. Nevertheless, the molecular mechanisms of these CoA-related actin abnormalities are not known.

Here we use *Drosophila* S2 cells to study mechanisms behind actin defects caused by CoA deficiency. We demonstrate that phosphorylation of a *Drosophila* homolog of cofilin, Twinstar (Tsr) [10], is increased in CoA deficient cells. Cofilin is an actin binding protein influencing depolymerization and severing of actin filaments and it plays an essential role in F-actin turnover [11, 12] and is has been reported that activity of cofilin

is inhibited by phosphorylation [13-15]. Further, we dissect the signaling pathways that lead to increased Tsr phosphorylation in response to disturbed CoA biosynthesis. Hence, this study reveals new levels of regulation of actin dynamics in *Drosophila*, in response to changes in CoA metabolism. Furthermore we show that regulation of cofilin in response to impaired CoA biosynthesis is conserved between *Drosophila* cells and human neuronal cells. By inhibiting PANK activity during neuronal differentiation *in vitro*, not only the phosporylation status of human cofilin is affected, but also the cellular morphology and neurite formation – process which are strongly dependent on actin remodeling.

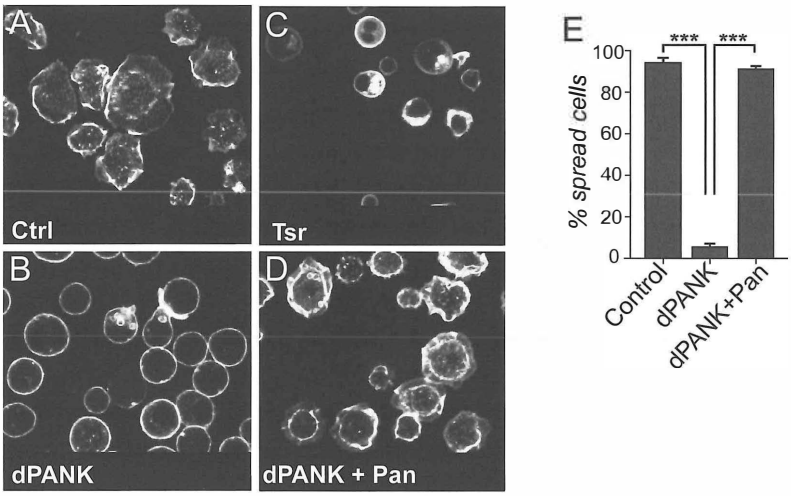


Figure 1. CoA deficient *Drosophila* S2 cells fail to form actin-based lamella.

A-D, S2 cells were plated on concanavalin A to induce cell spreading and lamella formation. Actin was visualized with rhodamine-phalloidin staining. Representative images of the Ctrl (A), dPANK/Fbl RNAi (B), Twinstar RNAi (C) and dPANK/Fbl RNAi-treated cells supplemented with pantethine (D) are shown. E, Quantification of the percentage of spread (lamella forming) cells as illustrated in A-D.

Results

dPANK/Fbl down-regulation leads to actin abnormalities in *Drosophila* S2 cells

In *Drosophila* pantothenate kinase is encoded by a single gene *dPANK/fbl* [8]. Decreased expression of dPANK/Fbl protein leads to a dramatic reduction in the levels of total CoA both in *Drosophila dPANK/fbl* mutant flies and Schneider's S2 cells [6]. Interestingly, *dPANK/fbl* mutant flies show actin abnormalities during spermatogenesis and oogenesis [8, 9]. To elucidate the link between CoA metabolism and actin cytoskeleton, we first investigated whether RNAi mediated down-regulation of dPANK/Fbl in S2 cells also leads to F-actin abnormalities. *Drosophila* Schneider's cells have been successfully used to identify new players involved in actin cytoskeleton dynamics and regulation of cell morphology [16, 17]. Hence, we plated control and dPANK/Fbl down-regulated S2 cells on concanavalin A-coated glass coverslips to induce cell flattening and spreading [16]. As revealed by phalloidin staining, control cells showed the characteristic morphology with lamella formation, whereas CoA deprived cells failed to undergo morphological changes under these conditions (Figure 1A and B, for quantification see Figure 1E). Impaired function of various proteins involved in formation of actin based lamella is associated with specific morphological changes in S2 cells [16]. We have compared the morphology of CoA deficient cells with these reported abnormalities and noticed striking similarities between dPANK/Fbl RNAi-treated cells and cells depleted of the *Drosophila* ADF/cofilin homolog, Twinstar (Figure 1C). Next, we investigated whether observed actin abnormalities where indeed the consequence of decreased cellular CoA levels or were due to the absence of dPANK/Fbl protein. Previously we showed that addition of pantethine to the cell culture medium restored CoA levels in the presence of strongly reduced levels of dPANK/Fbl protein [6](Siudeja et al., in press). Pantethine supplementation reversed the morphological abnormalities of dPANK/Fbl depleted cells (Figure 1D, for quantification see Figure 1E), confirming that decreased CoA levels are strongly associated with a failure of S2 cells to spread on concanavalin A coated surface and are strongly associated with abnormal actin based lamella.

Twinstar phosphorylation is increased in CoA-deficient cells

The similarity in morphology between dPANK/Fbl depleted and Tsr depleted cells prompted us to hypothesize that Twinstar could be involved in the actin abnormalities in the CoA deficient background. Tsr is essential for actin remodeling in *Drosophila*, both *in vivo* and *in vitro* [10, 16, 18, 19]. The actin binding and severing activity of Tsr is known to be inhibited by phosphorylation at the conserved serine residue 3. Thus, using specific antibodies [20], we investigated levels of phopsho-Twinstar in CoA-deficient S2 cells. Western blot analysis revealed a significant increase in phospho-Twinstar in

dPANK/Fbl RNAi-treated cells as compared to control cells (Figure 2A). Further, we used a non-genetic approach to block the CoA biosynthesis pathway by using the pantothenate kinase inhibitor HoPan [21]. HoPan treatment resulted also in an increase in phospho-Tsr levels in S2 cells (Figure 2B). Additionally, the increase in phosphorylation in both cases, after dPANK/Fbl RNAi and after HoPan treatment, was rescued by the addition of pantethine, demonstrating that, in S2 cells Twinstar phosphorylation levels are responsive to changes in CoA levels and do not respond to the levels of the PANK protein itself. Our morphological studies (Figure 1) and these studies on increased phospho-Twinstar (Figure 2) strongly suggest that decreased CoA levels are associated with hyperphosphorylation (and inhibition) of Twinstar, leading to the observed actin abnormalities.

Rho GTP-ase signaling is involved in Twinstar hyperphosphorylation associated with decreased CoA levels

Having established that CoA deficiency correlates with defects in F-actin organization and with an increase in Twinstar phosphorylation, we further aimed to identify upstream mediators of this response. Cofilin phosphorylation is catalyzed by a family of LIM kinases [13, 15]. In *Drosophila* two kinases belonging to this group have been characterized, dLimk - the ortholog of human LIM kinase [22] and Center divider (Cdi) - orthologus to human testicular kinase 1 (TESK1) [23]. Further, the reactivation of cofilin is achieved by dephosphorylation catalyzed by Slingshot (Ssh) phosphatases [14]. Hence, by using an RNAi-approach we tested whether the increased phosphorylation of Twinstar in a CoA-deficient background is mediated by the above mentioned players. Consistent with previous reports [20], RNAi induced silencing of either dLimk or Cdi resulted in a decrease in basal Twinstar phosphorylation levels, whereas a knockdown of Ssh phosphatase induced the opposite effect (Figure 3A). Interestingly, cells expressing reduced levels of dLimk still showed an increase in Twinstar phosphorylation in response to decreased CoA levels caused by HoPan treatment. Conversely, cells expressing reduced levels of Cdi kinase or Ssh did not show any change in the levels of phospho-Twinstar upon incubation with HoPan (Figure 3B and C). These results implicate that normal levels of both Cdi kinase and Slingshot phosphatase are required for increased phosphorylation of Twinstar in response to impaired CoA metabolism. The two-fold induction in Twinstar phosphorylation in the presence of reduced levels of Limk indicate that either the phosphorylation is dLimk independent or reduced levels of dLimk are still sufficient to induce Twinstar phosphorylation.

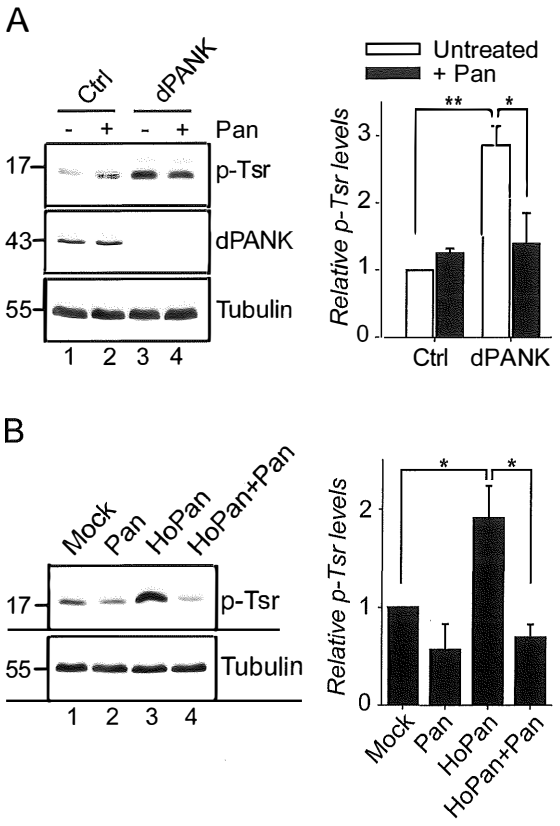


Figure 2. Inhibition of dPANK activity in S2 cells induces hyperphosphorylation of Twinstar.

Immunoblots of whole cell extracts were incubated with an antibody against phospo-Twinstar. Tubulin was used as a loading control. A, Cells were treated with control dsRNA or dsRNA to down-regulate dPANK/Fbl protein levels and medium was supplemented with pantethine when indicated. The graph illustrates the quantified levels of p-Twinstar relative to untreated control cells. B, S2 cells were cultured in the presence of pantethine, HoPan or both compounds. The graph illustrates the quantified levels of p-Twinstar relative to untreated control cells.

Rho GTP-ases are well known players in actin signaling. Rho, Rac and Cdc42 interact with a large number of downstream partners and herewith they induce coordinated changes in the organization of the actin cytoskeleton (reviewed in [1]). Using the same RNAi approach as described above we investigated the involvement of Rho GTP-ases in the regulation of Twinstar upon PANK inhibition. Consistent with previous reports [24], down-regulation of Rho1 (*Drosophila* Rho kinase) decreased basal Twinstar phosphorylation. Down-regulation of Rac1/2 or Cdc42 created the opposite effect (Figure 3D). Although effects of Rac1/2 and Cdc42 on Twinstar phosphorylation can vary between different cell types [25, 26], our results are consistent with some of these, showing that Rac1/2 or Cdc42 depletion causes increased phosphorylation of Twinstar in S2 cells [20]. When the RNAi–treated cells were subsequently treated with HoPan to inhibit PANK activity, no further increase in Twinstar phosphorylation was observed under these conditions, whereas Twinstar phosphorylation significantly increased after HoPan treatment in control cells. Thus, cells depleted of either Rho, Rac1/2 or Cdc42 maintained constant levels of phospho-Twinstar regardless of HoPan treatment (Figure 3D and E). All together these results suggest that in *Drosophila* S2 cells cofilin regulation in response to fluctuating CoA levels is mediated by multiple pathways, which include Cdi kinase and slingshot phosphatase activity, both of which are known regulators of the cofilin phospho-status.

PANK inhibition inactivates mammalian cofilin and affects neuritogenesis *in vitro*

In the experiments as shown above we demonstrate that interfering with the pantothenate kinase activity in *Drosophila* S2 cells results in the failure to form actin-based lamellipodia and phosphorylation (and most likely inactivation) of Twinstar – the *Drosophila* ortholog of cofilin. In humans mutations in the PANK2 gene cause PKAN – a severe neurodegenerative disorder with a largely unresolved pathophysiology. The regulation of cytoskeletal dynamics plays a fundamental role in neuronal function and it is especially important in the process of neuritogenesis [27, 28]. Furthermore, cofilin (together with Lim kinases and Slingshot) appears to be a key regulator of actin cytoskeleton dynamics during growth cone formation and neurite extension [29-33]. Therefore, we investigated whether inactivation of PANK activity in human neuronal cells leads to changes in the phosphorylation status of cofilin and whether this correlates with altered neuronal morphology. First, we cultured undifferentiated neuroblastoma SHSY-5Y cells during four doubling times with addition of HoPan in the cell culture medium in order to block the enzymatic activity of PANK. Under these conditions of HoPan treatment reduced cell counts and abnormal morphology were observed (Figure 4A and B). In addition, HoPan also induced a detachment of the treated cells from the culture dishes. This clearly indicates that, similarly to *Drosophila* S2 cells, impaired CoA biosynthesis affects survival and morphology of dividing human neuroblastoma cells.

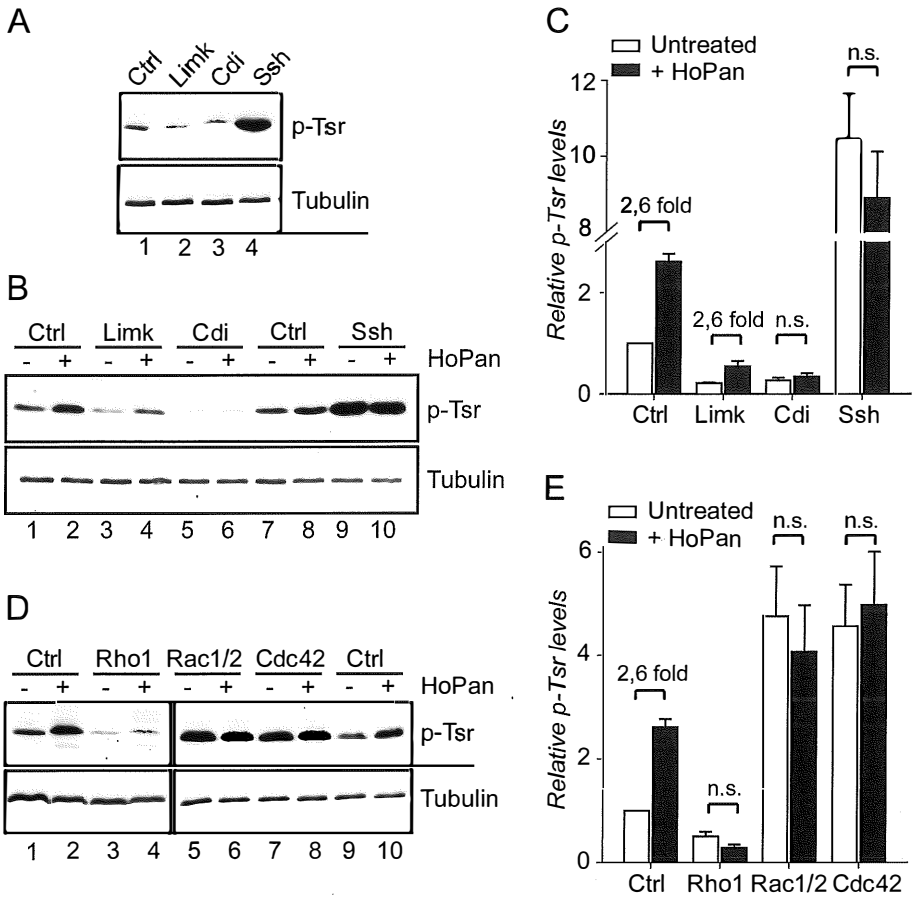


Figure 3. Rho GTP-ase signaling is involved in the regulation of Twinstar in response to decreased levels of CoA.

A, *Drosophila* LIM kinase (dLIMK and Center divider (Cdi)) and Slingshot phosphatase (Ssh) regulate levels of p-Twinstar in S2 cells. Cells were treated with dsRNA against dLIMK, Cdi, Ssh or with control dsRNA and the levels of p-Twinstar were assayed using Western blot analysis. Tubulin was used as a loading control. B, dLIMK, Cdi or Ssh were down-regulated for 3 days after which cells were subcultured and left untreated or HoPan was added to the cell culture medium. After additional 48 hr cell lysates were assayed for Twinstar phosphorylation. C, Quantification of the levels of p-Twinstar as illustrated in B. Fold change after HoPan addition is indicated above the bars, n.s: not significant. D, Rho1, Cdc42 down-regulation or Rac1-2 double down-regulation was performed for 3 days, after which cells were subcultured and left untreated or HoPan was added to the cell culture medium. After additional 48 hr cell lysates were assayed for Twinstar phosphorylation. E, Quantification of the levels of p-Twinstar as illustrated in D. Fold change after HoPan addition is indicated above the bars, n.s: not significant.

Further we used retinoic acid (RA) to differentiate SHSY-5Y cells [34, 35] and to investigate the effects of HoPan on cell spreading and neurite formation. First we examined cofilin phosphorylation levels upon differentiation of SHSY-5Y cells. Western blot analysis revealed that treatment with RA resulted in a significant decrease in cofilin phosphorylation as compared to mock treated cells (Figure 5A). Further, we compared the levels of phospho-cofilin in differentiated SHSY-5Y cells non-treated or treated with HoPan. Similarly to our results obtained with *Drosophila* cells, HoPan induced an increased phosphorylation of cofilin in human SHSY-5Y cells (Figure 5B). Finally, we investigated the ability of differentiated cells to spread and to form neurites on a poly-D-Lysine (PDL)-coated surface. 20 hours after seeding on PDL most of the HoPan treated cells remained only loosely attached to the surface exhibiting a round up morphology. On the contrary, under these conditions more than 80 % of control cells were spread on PDL (Figure 5C). We performed a rhodamine-phalloidin staining to visualize F-actin (Figure 5D), which illustrated the efficient spreading of control cells, with highly organized actin filaments and formation of growth cones. However, in contrast to the control cells, most of the HoPan-treated SHSY-5Y cells displayed rounded morphology with some accumulation of F-actin around the rims of the cells, a phenotype similar to the one observed in dPANK/Fbl-depleted *Drosophila* S2 cells (Figure 1B). To assess the formation of neurites, 48 hr after plating, differentiated cells were fixed and immunolabeled with an antibody against beta-III-tubulin – a neuronal specific isoform of tubulin [36-38]. Under these conditions around 60 % of control cells formed neurites, whereas after HoPan treatment the percentage of neurite-bearing cells was less than 10 % (Figure 5E and F). Finally, we tested whether the HoPan-induced phenotypes in SHSY-5Y cells could be reverted by the addition of pantethine to the cell culture medium. The rescue by pantethine was evident for all the phenotypes investigated (Figure 5 B-F), strongly suggesting that the observed defects are due to decreased levels of CoA and not due to some non-specific (and non-PANK related) effect of HoPan. These results indicate that phosphorylation (and most likely inactivation) of cofilin under conditions of impaired CoA biosynthesis coincides with impairment in cell spreading and neurite formation *in vitro* –processes which are strongly dependent on actin remodeling.

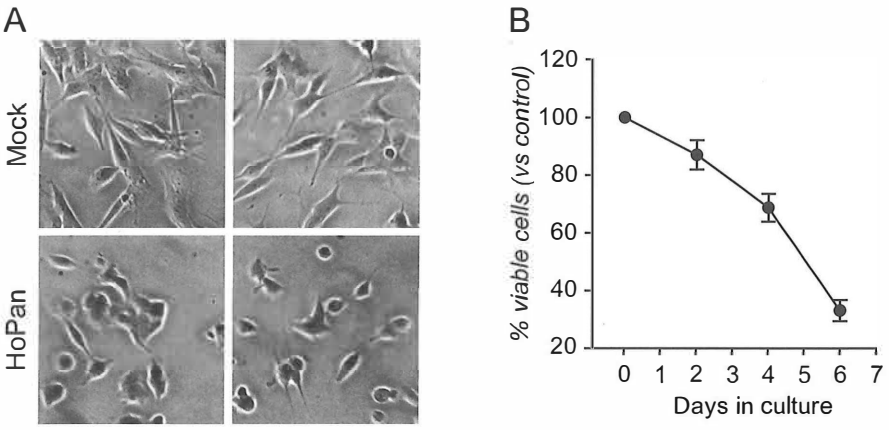
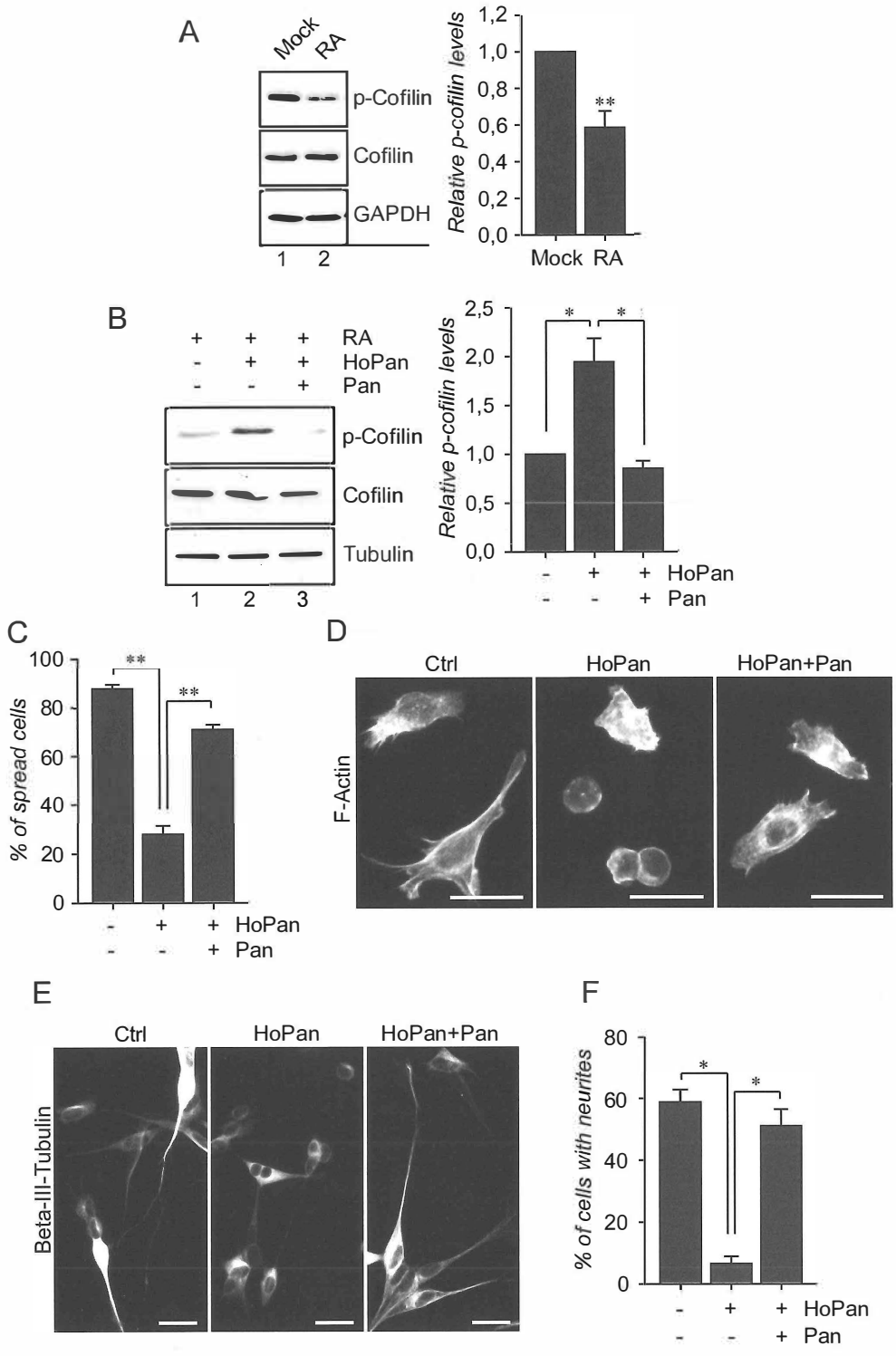


Figure 4. The morphology and viability of cultured human neuroblastoma SHSY-5Y cells is affected upon PANK inhibition.

SHSY-5Y cells were cultured in medium supplemented with 0,5 mM HoPan. A, After 6 days in culture the cell morphology was visualized with an inverted microscope. Representative images of control (mock treated) and HoPan treated cells are shown. B, Cell viability after 0-6 days in culture with HoPan was assayed by a Trypan blue exclusion test. Viability of control untreated cells was set as 100 % at each time point.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◀ Figure 5. HoPan induces hyperphosphorylation of cofilin and affects neurite formation in neuronal differentiation *in vitro*.

A, Undifferentiated and retinoic-acid (RA) differentiated SHSY-5Y cells were lysed and levels of total and phosphorylated-cofilin were assayed using Western blot analysis with specific antibodies. Representative blots are shown. GAPDH was used as an additional loading control. Levels of p-cofilin were quantified from 2 independent experiments. The p-cofilin/cofilin ratio was set as 1 in mock treated cells. B, RA-differentiated SHSY-5Y cells were additionally treated with HoPan or HoPan and pantethine. Levels of p-cofilin were assayed and quantified as in A. C-F, HoPan or HoPan plus pantethine-treated cells were differentiated with RA and plated on poly-D-lysine. After 24 hours, cells were investigated for their efficiency to spread on the PDL surface (C) and simultaneously cells were fixed and stained with rhodamine-phalloidin to visualize F-actin (D). 48 hr after seeding on PDL cells were fixed and stained for beta-III-tubulin (E). The percentage of neurite-bearing cells was calculated for each condition (F). Scale bars represent 25 µm.

Discussion

The actin cytoskeleton mediates a variety of essential biological functions in all eukaryotic cells. The molecular mechanisms responsible for actin remodeling have been extensively studied. However, relatively little is known how cellular metabolism influences cell morphology and cytoskeleton dynamics. Recent studies convincingly show that these events are closely linked. For example, it is well recognized that insulin and growth factor signaling induces actin filament remodeling promoting membrane ruffling in diverse mammalian cell types [39, 40]. In *Drosophila* S2 cells F-actin rich lamellapodia are also being formed in response to insulin-induced Akt signaling [20]. Further, studies using *Drosophila* and mammalian systems suggest that energy metabolism is directly linked to cell structure regulation via the Lkb1/AMP-activated protein kinase (AMPK) signaling pathway [41-44]. The physiological importance of this crosstalk between metabolism and the cytoskeleton remains poorly understood but relevant to investigate, because it may contribute to the understanding of specific pathological conditions. For example Lkb1, which plays a central role in energy metabolism and actin filaments assembly [45] is linked to tumorigenesis. Mutations in the human *LKB1* gene result in increased incidence of epithelial cancer [46], which suggests that loss of Lkb1 in epithelia may contribute to the tumorigenic process through effects on the actin cytoskeleton. Disturbance of the tight link between the cellular metabolic status and regulation of the actin cytoskeleton may also underlie a number of metabolic diseases with an unresolved pathophysiology.

PKAN is a severe neurodegenerative disease caused by mutations in the human pantothenate kinase 2 (PANK2)-encoding gene and the molecular basis of this devastating disease are largely unknown [4]. Previously we have established *Drosophila* and human cell line models for PKAN, in which PANK activity is impaired [5, 6]. Here, we demonstrate that impaired CoA metabolism influences actin associated cellular evens, such as the regulation of the cell shape. We show that inhibition of PANK activity results in changes in cell morphology in *Drosophila* as well as in human neuronal cell cultures. Further we identify a conserved mechanism of cofilin inactivation in response to decreased CoA levels. We show that in *Drosophila* the inability of CoA-deficient S2 cells to form actin rich lamellipodia, correlates with an increase in phospho-cofilin levels. These results underscore previously published data that in general cofilin is an important regulator of actin dynamics (reviewed in [47]) and are in agreement with data showing that increased cofilin phosporylation is associated with the inability of S2 cells to form actin-based lammelipodia [20].

Finally, in our current work we show that not only *Drosophila* cells but also differentiated human neuronal cells show abnormal F-actin organization and an increase in cofilin phosphorylation upon impaired PANK function. Differentiated neuroblastoma SHSY-5Y cells undergo extensive morphological changes, which finally lead to the formation of neurites [35] . During these morphological changes, we observed that cofilin is maintained in a non-phosphorylated (active) state. The latter is most likely required for high actin filament dynamics, necessary to undergo the morphogenesis. Analogous involvement of cofilin in neuronal differentiation has also been reported both in primary cultures (such as isolated chicken neurons) and in other cell lines (such as PC12 cells) [29-33]. We show that the addition of PANK inhibitor, HoPan, to the medium not only inactivates cofilin but also affects the ability of differentiated SHSY-5Y cells to interact with tissue culture surface and the ability to form neurites.

The physiological importance of actin regulation in neuronal function *in vivo* can be illustrated by a contribution of actin signaling pathways to brain disorders, especially to mental retardation [48, 49]. Moreover cofilin activity itself has recently been reported to regulate neuronal migration [50], spine morphology [51] and synaptic plasticity [52] in mice. In humans, abnormal expression of LIM-kinase1 is associated with a mental disorder with profound cognition deficits [53]. Together these and our studies underscore the physiological importance of actin regulation and suggest that mutations that disrupt normal PANK activity (as in PKAN) can lead to altered actin dynamics and neuronal dysfunction. It needs to be pointed out that altered CoA metabolism does also influence other cytoskeletal components because recently we demonstrated that tubulin acetylation is strongly decreased in *Drosophila* and human cell models of PKAN (Siudeja et al., in press). However, cell spreading and neurite formation (as measured here) depend mostly on the reorganization of the actin cytoskeleton, whereas microtubule polymerization is required for the elongation of already existing neurites [27, 54]. Additional studies will be required to investigate whether cofilin inactivation and/or actin abnormalities indeed underlie the pathology of PKAN, although at present these experiments are hindered by a very limited access to patient derived material.

Surprisingly little is known about the implications of altered *de novo* CoA synthesis in higher eukaryotes and our results reveal a novel and conserved link between CoA metabolism and the regulation of actin dynamics. We address, for the first time, the consequences of impaired PANK function on actin organization and dynamics and the results obtained contribute to the general cell biology knowledge and may potentially increase our understanding of the human neurodegenerative disorder PKAN.

Materials and Methods

Drosophila cell culture - *Drosophila* Schneider's S2 cells were maintained at 25°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10 % heat inactivated fetal calf serum (Gibco) and antibiotics (penicillin /streptomycin, Invitrogen). Cells in the exponential phase of growth were used for all the experiments. When required 0,5 mM HoPan (Zhou Fang Pharm Chemical, Shanghai, China) and/or 0,1 mM pantethine were added directly to the cell culture medium. HoPan treatment was continued for 2-3 days.

dsRNA synthesis and RNAi - T7-flanked primer sequences were designed to amplify gene specific DNA templates (for primer sequences, see Supplementary Table S1). DNA templates were amplified using standard PCR methods. Double-stranded RNAs were obtained by *in vitro* transcription and purified using MegaScript RNAi Kit (Ambion). 2 pmols of DNA templates were used per 20 µL *in vitro* transcription reaction. dsRNA treatment was carried out as described previously [55]. Cells were incubated in serum-free medium containing 40nM dsRNA for 1 hour, following the addition of serum containing medium. The cells were incubated for 3 days to induce an efficient knock-down. Cells were subcultured, drugs (HoPan and/or pantethine) were added to the medium and the cells were maintained for additional 2 – 3 days until analysis.

SHSY-5Y culturing and differentiation - SHSY-5Y human neuroblastoma cells were maintained in dMEM (Invitrogen) supplemented with 10 % FCS (Gibco) and antibiotics (penicillin /streptomycin, Invitrogen). Cells of a passage between 10-30 were used. For HoPan treatment, cells were cultured in custom made dMEM without vitamin B5 (Thermo Scientific) supplemented with dialyzed FCS (Thermo Scientific). This medium did not affect normal growth of the cells. Cells were differentiated with 50 µM retinoic acid (RA, Sigma) for 48-72 hours. Differentiated SHSY-5Y cells were plated in poly-D-Lysine coated culture dishes and cell spreading and, neurite formation were quantified after 20 hours and 48 hours respectively.

Immunoblotting - Equal numbers of *Drosophila* S2 cells were centrifuged, washed once with PBS and lysed immediately in 1 X Laemmli Sample Buffer (62,5 mM Tris/HCl pH 6,8; 2 % SDS; 10 % glycerol; bromophenol blue) with 10 mM NaF. SHSY-5Y cells were washed 1 time with PBS in tissue culture dishes and removed from the surface by scraping the cells into PBS. After centrifugation cells were lysed in 1 X Laemmli Sample Buffer with 10 mM NaF. All sample were further sonicated and boiled for 4 min with 5 % β-mercaptoethanol. Protein extracts were run on 12,5 % SDS-Page gels, transferred onto nitrocellulose membranes and blocked with 5 % milk in PBS/0,1 % Tween, following by an overnight incubation with primary antibodies. The following antibodies were used: rabbit anti-dPANK/Fbl [5], rabbit anti-Twinstar [20], mouse anti-rho1 (p1D9, Developmental Studies Hybridoma Bank), rabbit anti-cofilin and rabbit anti-phospho-

cofilin (Cell Signaling) and mouse anti-tubulin (Sigma). HRP-conjugated secondary antibodies (Amersham) were used and detection was performed using enhanced chemiluminescence. Band intensities were quantified with Adobe Photoshop.

Immunofluorescence - For immunofluorescence *Drosophila* S2 cells were seeded on concanavalin A-coated glass microscope slides and allowed to spread for 45 min. SHSY-5Y cells were plated on poly-D-lysine-coated glass slides and cultured for 48 hours. Cells were fixed with 3,7 % formaldehyde for 15 minutes, washed 3 times with PBS and permeabilized with 0,2 % Triton X-100 for 10 minutes. Slides were further blocked with 3 % BSA in PBS/0,1 % Tween for 30 minutes, followed by the 2 hour-incubation with primary antibody (mouse anti-beta-III-tubulin (clone 2G10, Sigma)). Secondary goat anti-mouse-Alexa488 antibodies (Molecular Probes) were used according to the manufacturer instructions (1 hour incubation). To visualize filamentous actin, cells were stained for 20 minutes with 20 U/mL rhodamine phalloidin (Molecular Probes) diluted with 3 % BSA in PBS/0,1 % Tween. Finally, microscope slides were mounted using CitiFluor mounting medium (Citifluor Ltd).

Fluorescent microscopy and image quantification - Fluorescent images were obtained with a Leica fluorescent microscope and processed with Leica software and Adobe Photoshop. More than 150 cells (from at least 10 different microscopic fields) per condition were counted to quantify the cell spreading (S2 cells) and neurite formation (SHSY-5Y cells).

Statistical analysis - All results were obtained in at least two independent experiments, with a duplo or triplo of each experimental condition. Statistical significance was calculated using the Student's t-test (two-tailed, with equal variance). p values $< 0,05$ were considered significant and were indicated as follows: $p < 0,05$ - *; $p < 0,01$ - **; $p < 0,001$ - ***. Graph error bars represent S.E.M.

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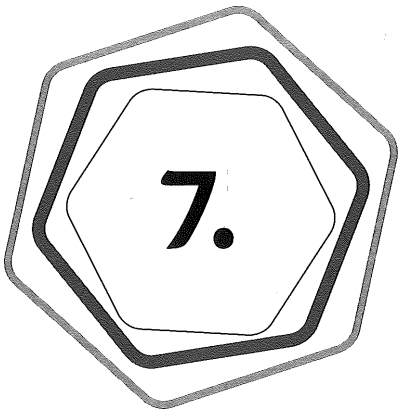
Supplementary Information

Gene	CG number	Primers
dPANK/Fbl	CG5725	fwd - CGTGATACGCACCTACAGATG
		rv - GCCATTGGACCAGAACTCCAT
dLimk	CG1848	fwd - ATGTCAGCGTCCACGTTAATC
		rv - GGTCACCTTGAAAACCTTGC
Cdi/dTESK	CG6027	fwd - TTCGGACGGCAGCTGAACGG
		rv - GCTGAGCAACGCATGGTGGC
Ssh	CG6238	fwd - GATGTTGGACGCCATGAAG
		rv - CCTCGAGATCGTGAATTCGT
Rho1	CG8416	fwd - TTAATAACAGCCCTGCGCTC
		rv - TTGGTGATTTCACAACACTCAG
Rac1	CG2248	fwd - CGGTCACACTGCAGTACACA
		rv - CACGACGACGCACTTGAT
Rac2	CG8556	fwd - AGCTGAGCTGCGATACAAGG
		rv - GGAAGGCGTTGGTCGTATAG
Cdc42	CG12530	fwd - CTATCCGCAGACGGATGTC
		rv - GCGTGTGAGGAAAGCAGAAT

Table S1. The following primers were used to generate dsRNAs. 5' T7 RNA polymerase binding site (TAATACGACTCACTATAGGG) was proceeding each primer's sequence.



7.

Summarizing discussion and perspectives

Summarizing Discussion

CoA at crossroads of metabolic pathways

A wide range of metabolites participate in the cellular intermediate metabolism when nutrient compounds are utilized and converted into cellular components. Carbohydrates (glucose), lipids, amino acids and nucleic acids are continuously broken down and remodeled by cells to form countless metabolic intermediates and byproducts. Especially some of these intermediate compounds occupy important positions at crossroads of many metabolic pathways and as such they constitute important signaling molecules that effect cell fate in multiple ways [1]. One of these central metabolites, which link multiple biochemical pathways, is acetyl-CoA. Because acetyl-CoA is a thioester between CoA and acetic acid, CoA metabolism itself is also of central importance.

Figure 1 depicts a simplified scheme of "textbook" cellular acetyl-CoA metabolism. When glucose enters the cell it is converted to pyruvate in the glycolytic pathway. Pyruvate produced by glycolysis in the cytosol is further decarboxylated and combined with CoA to form acetyl-CoA. Acetyl-CoA may then enter the TCA cycle, ultimately resulting in production of ATP and NADH molecules. NADH is then used in mitochondrial oxidative phosphorylation – one of the most important energy producing processes in the cell. In addition to participating in the mitochondrial TCA cycle, acetyl-CoA participates in a number of cytosolic as well as nuclear events. Acetyl-CoA cannot cross the inner mitochondrial membrane, but intramitochondrial acetyl-CoA is combined with oxaloacetate to form citric acid, which is transported out of the mitochondria. The cytosolic CoA pool is then necessary to resynthesize acetyl-CoA from citrate. A similar conversion from transportable citrate to acetyl-CoA also takes place in the nucleus. Cytosolic acetyl-CoA is primarily used for the synthesis and elongation of fatty acid chains. However, acetyl-CoA acts also as a precursor for posttranslational modification of cytosolic and nuclear proteins. By these means acetyl-CoA not only directly participates in the reactions of the intermediate metabolism, but also reversibly influences the activity of many proteins possessing a regulatory, structural or enzymatic function.

CoA acts as a carrier of an acetyl group (acetyl-CoA), but it also reacts with other acyl groups of different carbon chain lengths. All together, CoA and its thioesters are used as a substrate by around 4 % of all cellular enzymes (reviewed in [2]) and a detailed review of all the biochemical pathways in which CoA thioesters are involved falls outside the scope of this chapter. Although the biochemical function of CoA has now been known for over 60 years and most of the biochemical reactions in which CoA plays an important role have already been well described, relatively little is known how impaired CoA metabolism influences cellular and whole-organism functioning. Aberrations in metabolic pathways and their downstream effects are known to contribute to the etiol-

ogy of many disease, including cancer and degenerative disorders [1]. Many inborn errors of metabolism result from deficiencies of one of the enzymes acting on acyl-CoAs [3]. Here we will discuss the consequences of impaired CoA biosynthesis addressed in this thesis and how these could potentially relate to pantothenate kinase-associated neurodegeneration (PKAN) – a disease caused by an inborn defect of the CoA biosynthesis pathway in humans [4, 5].

Alternative pathway of CoA biosynthesis – pantethine restores CoA levels independently of pantothenate kinase

In chapter 4 of this thesis we show genetic evidence for the existence and, more importantly, the physiological importance of a pathway parallel to the canonical *de novo* CoA biosynthesis. This pathway uses pantethine (instead of pantothenic acid) as a precursor for CoA biosynthesis. Although the biochemical conversion of pantethine into CoA has already been described before [6], it was assumed that pantothenate kinase (PANK) activity constitutes an essential step of the pathway, as it does in case of pantothenic acid conversion. However, we show that feeding pantethine restores CoA levels in *dPANK/fbl* mutant flies as well as in dPANK/Fbl-depleted *Drosophila* S2 cells. Thus, we demonstrate that pantethine can be used as a substrate for the *de novo* biosynthesis of CoA in the absence (or at least in the presence of severely reduced levels) of PANK.

Pantethine supplementation proves very efficient because it improves or completely rescues almost all the phenotypes (discussed in the following paragraphs) induced by PANK-deficiency. Therefore this indicates additionally that the phenotypes observed and reported by us are induced by decreased levels of CoA and not by the absence of PANK protein. In *in vitro* assays, pantethine restores completely cell proliferation rates, impaired mitochondrial function (chapter 4), impaired protein acetylation (chapter 5) and abnormal F-actin dynamics (chapter 6) in dPANK/Fbl-depleted *Drosophila* S2 cells. In vivo, feeding pantethine to flies improves survival, locomotor function as well as mitochondrial structure and activity (chapter 4). Although male and female infertility, another phenotype characteristic for *dPANK/fbl* mutant flies, is not rescued upon pantethine feeding (unpublished observation), this may be due to the fact that CoA levels may not be fully restored in the reproductive organs. In experiments as described in chapter 4 pantethine was fed only to adult flies, thus it is highly probable that pantethine fails to rescue fertility because reproductive organs are affected by low levels of CoA already during larval development. Further experiments are necessary to address these possibilities.

All together we show that pantethine is a promising compound and can ameliorate the PKAN-like phenotypes. This knowledge may be of great relevance for designing future therapies directed against PKAN.

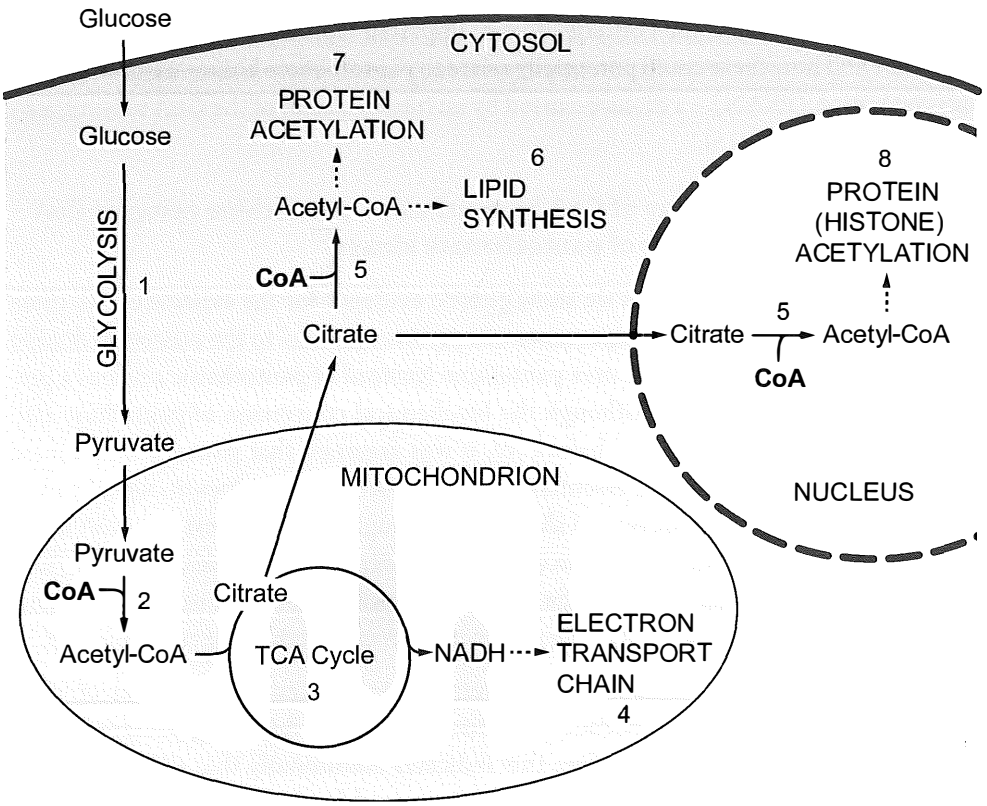


Figure 1. Overview of metabolism – CoA and acetyl-CoA as central metabolites.

Selected metabolic pathways central to the metabolism of CoA and acetyl-CoA are shown. Glucose is transported into the cell and enters the glycolytic pathway (1). Glycolysis-derived pyruvate is shuttled to mitochondria and is further converted to acetyl-CoA (2). Mitochondrial acetyl-CoA feeds into the TCA cycle, which ultimately produces ATP and NADH (3). NADH is then used in mitochondrial oxidative phosphorylation for ATP production (4). Intramitochondrial acetyl-CoA has to be converted to citrate in order to be transported into the cytoplasm and the nucleus. In presence of the cytosolic or nuclear CoA citrate is converted back to acetyl-CoA (5). Cytosolic acetyl-CoA is used for lipid synthesis (6) and protein acetylation (7). Acetyl-CoA-dependent protein acetylation takes also place in the nucleus and is especially abundant on histone proteins (8).

Coenzyme A and mitochondrial function

The synthesis of CoA takes place in the cytoplasm (see chapter 1 for more details on the CoA biosynthesis pathway). However PANK2, an isoform of human pantothenate kinase which is mutated in PKAN, is the only enzyme in the CoA biosynthesis pathway which localizes to the mitochondria [7-9]. This unique localization of PANK2, together with the fact that CoA concentrations are the highest in mitochondria among different cellular compartments [10], may suggest that mitochondrial dysfunction could be one of the primary consequences of impaired CoA metabolism.

Data presented in this thesis indeed illustrate that *dPANK/fbl* mutant flies as well as dPANK/Fbl-depleted *Drosophila* S2 cells have severe mitochondrial defects (Chapter 4). In flies at the morphological level impaired CoA biosynthesis coincides with damaged mitochondria in flight muscles. This structural damage is reflected by a decrease in functionality of isolated mitochondria, as measured by the membrane potential-sensitive JC-1 assay. Similarly, reduced mitochondrial function is observed in *Drosophila* S2 cells after down-regulation of dPANK/Fbl by RNAi. Finally, down-regulation of PANK2 in human HEK293 cells leads also to impaired mitochondrial activity. These results are in agreement with previously published data, describing HoPan feeding to mice in order to inhibit PANK activity. The livers from HoPan-treated mice exhibit a distinct pathology characterized by severely swollen mitochondria [11]. All together, these findings prove that hampered CoA metabolism indeed is associated with impaired integrity and function of mitochondria.

The human PANK2 isoform which is mutated in PKAN is localized to mitochondria [7-9]. Contrary to mammals, *Drosophila* has only one PANK gene, however it encodes multiple isoforms, one of which is also targeted to mitochondria [12]. Interestingly, Wu et al have recently reported that in flies only this mitochondrially-targeted dPANK/Fbl isoform as well as mitochondrially-localized human PANK2 can fully rescue the phenotypes of dPANK/Fbl mutants [12]. This finding may indicate that mitochondrial and not cytoplasmic localization of pantothenate kinase activity is essential in flies and when disturbed it results in PKAN-like characteristics. However, it needs to be noted that no experimental data exists on the enzymatic activity of the non-mitochondrial isoforms of dPANK/Fbl. Wu et al measured a decrease in pantothenate kinase activity in dPANK/Fbl mutants and an increase in PANK activity upon overexpression of mitochondrial dPANK/Fbl isoform, however they did not measure the enzyme activity upon overexpression of other isoforms, which fail to rescue dPANK/Fbl mutant flies.

All together, it becomes clear that impaired CoA biosynthesis alters mitochondrial function and it is possible that mitochondrially localized pantothenate kinase activity is of crucial importance for cellular homeostasis. Abnormalities in mitochondrial function are associated with a number of neurodegenerative diseases [13]. Since mitochondria are the energy factories of the cell, tissues of high energy demands (such as brain)

may be the first to suffer from impaired mitochondrial function. Until now it has not been convincingly proven if mitochondrial defects indeed underlie the pathophysiology of PKAN, however, based on our data and the data of others, this hypothesis is highly probable.

Role of the CoA biosynthesis pathway in protein acetylation

In this thesis we describe not only the consequences of impaired CoA metabolism on the level of mitochondrial functioning, but we also address a more unexpected link between decreased CoA levels and impaired protein acetylation.

Lysine acetylation is, after phosphorylation, the most common and the most intensively studied posttranslational modification of polypeptide chains. Numerous acetyl transferases catalyze the transfer of an acetyl group on lysine side chains and acetyl-CoA serves as a substrate for this reaction (a donor of the acetyl group). In particular, acetylation of nuclear histones has been extensively studied as a major posttranslational modification which plays an important role in transcriptional regulation but also in DNA replication and DNA repair (reviewed in [14]). In addition to histone acetylation many non-histone and non-nuclear proteins have been identified to undergo reversible lysine acetylation [15, 16]. Together with these recent discoveries of how complex the acetylome is, comes the understanding that acetylation plays a major role in the regulation of cell metabolism and function.

In chapter 5 of this thesis we address the question whether impaired CoA metabolism influences protein acetylation. CoA acts as a carrier of the acetyl group and we found that decreased CoA levels result in impaired acetylation homeostasis. Using RNAi or biochemical approaches (chemical inhibition) we demonstrate that impaired PANK activity affects nuclear acetylation of histones as well as cytoplasmic acetylation of tubulin. This holds true for *Drosophila*, *C. elegans* as well as human tissue culture models of PANK deficiency, which underscores the conserved role of CoA metabolism in protein acetylation. These data are consistent with already published reports. A major source of cytoplasmic and nuclear acetyl-CoA in eukaryotic cells is the conversion of citrate catalyzed by ATP-citrate lyase (ACL) (see Figure 1, marked with (5)). Alternatively, acetyl-CoA can also be generated from acetate by acetyl-CoA synthetase (AceCS). Both reactions require CoA as a substrate. Inhibition of either ACL or AceCS leads to impaired acetylation of histones [17-19], however, in contrast to our data, acetylation of non-histone proteins seems not to be affected under these tested conditions. This could be explained by the fact that in the presence of decreased CoA levels, as in our studies, both of the acetyl-CoA-producing pathways are blocked, resulting in a stronger reduction of acetyl-CoA levels. From our observations it is clear that acetylation of histones responds the fastest to changing CoA levels, while changes in tubulin acetylation are more difficult to detect. On the other hand, this may further imply that acetylation of other

proteins may also be reduced upon CoA deficiency, though more sensitive techniques (like mass spectrometry) are required to test this hypothesis. Furthermore, acetyl-CoA serves as a substrate not only for acetylation of lysine residues, but also for N-terminal protein acetylation. Although N-terminal acetylation is much less extensively described than lysine acetylation, it is known that most eukaryotic proteins are N-terminally acetylated, which is thought to assist in proper function, localization and stability of proteins [20]. Interestingly, Yi et al have recently demonstrated that the levels of acetyl-CoA directly influence N-terminal protein acetylation, which further regulates a cell's sensitivity to apoptosis [21]. Thus, it cannot be excluded that impaired CoA biosynthesis may also affect N-terminal protein acetylation (in addition to lysine acetylation).

It is now well established that the acetylation/de-acetylation balance is greatly impaired under neurodegenerative conditions. Especially the acetylation of histones and tubulin has been linked to brain abnormalities during neurodegeneration or during age dependent memory impairment (reviewed in [22]). Treatment with histone deacetylase inhibitors (HDACi), meant to restore acetylation levels, was shown to ameliorate several neurodegenerative conditions in animal and cell culture models, including polyglutamine diseases [23-25] or Parkinsosn's disease [26]. Thus, our results of impaired histone and tubulin acetylation in presence of decreased PANK activity (Chapter 5), as in PKAN related neurodegeneration, may be of clinical relevance. We demonstrate that impaired histone and tubulin acetylation correlates with mutations in *dPANK/fbl* and HDACi can be used to rescue the levels of acetylated histones and acetylated tubulin in a PANK-deficient background. Moreover, HDACi treatment has a beneficial effect on the survival and locomotor function of *dPANK/fbl* mutant flies as well as on the survival of dPANK/Fbl-depleted *Drosophila* S2 cells exposed to genotoxic stress. Future experiments could concentrate on investigating further functional consequences of impaired acetylation in a PANK-deficient background. In particular, it would be interesting to test whether PANK inhibition results in impaired stability of axonal microtubules or impaired axonal transport in neurons, as these processes are essential for brain function and were both shown to depend on the acetylation of tubulin [27].

Impaired CoA biosynthesis and actin remodeling

Abnormal F-actin dynamics were reported in *dPANK/fbl* mutant flies during spermato- and oogenesis [28, 29]. This reveals an intriguing link between CoA metabolism and the regulation of the actin cytoskeleton. Interestingly, in a yeast two hybrid screen dPANK/Fbl was identified as an interacting partner for Twinstar (Tsr), a *Drosophila* ortholog of the actin depolymerizing factor cofilin [30]. Additionally, twinstar mutant flies share some of the phenotypes characteristic for *dPANK/fbl* mutants, such as abnormal cytokinesis or patterning defects [31-33]. This suggests that changes in actin dynamics may underlay phenotypes observed in a CoA-deficient background. Although we were not able to confirm the physical interaction between dPANK/Fbl and Tsr in

co-immunoprecipitation assays (data not shown), we show that dPANK/Fbl-deficiency results in the hyperphosphorylation of Tsr in cultured cells (Chapter 6). Cofilin (and Twinstar) phospshorylation is conserved between species and upon phosphorylation their actin severing activity is inhibited, leading to impaired actin dynamics [34, 35]. Thus, even though it is unlikely that dPANK/Fbl directly modulates Tsr activity (lack of physical interaction), we propose that a cross-talk between CoA metabolism and actin remodeling is mediated by posttranslational regulation of cofilin. Moreover, the changes in phospho-Tsr levels are indeed responsive to CoA levels, rather than a sole presence or absence of dPANK/Fbl protein, since an addition of pantethine to dPANK/Fbl-depleted cells restores normal phospho-Tsr levels and rescues the morphological defects in *Drosophila* S2 cells and in human neuroblastoma-derived cells.

Further studies should be directed towards identifying the upstream mediators of increased Tsr phosphorylation upon CoA deficiency. We provide evidence that a conserved cofilin kinase/phosphatase system is involved, as well as GTP-ases known to regulate actin dynamics in response to many different stimuli (Chapter 6). However, the challenge remains to identify the most upstream signaling events that lead to the regulation of cofilin in response to impaired PANK activity. Due to the fundamental role of CoA in the energy metabolism of the cell, the involvement of one of the central energy and growth factor-regulated proteins/pathways is highly probable. Previously published reports imply that the activity of cofilin can be regulated by the PI3 kinase/Akt pathway [36], S6 kinase [37] as well as by AMP-activated protein kinase [38]. Possibly, one (or more) of these pathways mediates the changes in cofilin phosphorylation in response to CoA depletion.

Regardless of the upstream players involved in the link between PANK and cofilin activity, our data demonstrate a conserved mechanism of cofilin hyperhosphorylation upon PANK deficiency in *Drosophila* and human neuronal cells. In differentiated human SHSY-5Y cells increased levels of phospho-cofilin that occur after chemical inhibition of PANK coincide with impaired cell spreading and impaired formation of neurites. Both processes are essential for neuronal function and highly dependent on actin remodeling events [39]. Cofilin has already been shown to regulate neuronal differentiation *in vitro* [40-42] as well as neuronal migration and synaptic plasticity *in vivo* [43, 44], and, in general, the involvement of actin regulation in brain function is well documented [45, 46]. Thus it is likely, that abnormal F-actin dynamics caused by decreased PANK activity may contribute to the neurodegenerative phenotype as observed in PKAN.

Concluding Remarks and Perspectives

Although the biochemical function of CoA is known for over 60 years, relatively little is known about the biological consequences of impaired CoA *de novo* biosynthesis. However, due to the involvement of CoA in a large number of biochemical reactions, the consequences of CoA deficiency are most likely multidirectional. Studies in *Drosophila*, mice and human cultured cells strongly indicate that the impairment of mitochondrial function may be a primary consequence of decreased cellular CoA levels. This in turn will lead to defects in the TCA cycle and in the oxidative phosphorylation, resulting in energy loss. Furthermore, previous reports demonstrate that impaired CoA metabolism leads to abnormalities in lipid metabolism [47]. This is understandable taking into account that acetyl-CoA is required to synthesize phosphatidic acid (PA), which is a main precursor for many lipids, and CoA is also necessary to support β-oxidation of fatty acids. Additionally, we now show that impaired CoA metabolism has further consequences on the level of structural organization of the cell as well as on the maintenance of genomic integrity. Cystoskeleton organization is responsive to CoA levels via cofilin-mediated changes in F-actin organization as well as via acetylation of microtubules which is directly dependent of acetyl-CoA levels. Acetylation on histones, on the other hand, links CoA metabolism with the epigenetic regulation of the genome. Thus, a complex network of interconnected pathways emerges, which all are affected when CoA *de novo* biosynthesis is impaired.

Apart from gaining a basic understanding of cellular biology, studying impaired CoA biosynthesis may contribute to the understanding of the pathophysiology of PKAN. Through this chapter we have discussed how the observed consequences of impaired PANK function could potentially contribute to the development of PKAN. These observations may in the future serve as starting points for the development of new therapies against this devastating disease. However, it needs to be noted that a substantial number (around 30 %) of the disease-associated mutations in human PANK2 do not affect the processing, mitochondrial localization, activity or stability of the protein [9, 48]. This would suggest that PKAN is not solely due to the loss of PANK2 activity. One could speculate that PANK2 posses another cellular function which is independent from pantothenate kinase activity. The primary structure (sequence) of PANK2 does not suggest that the protein itself could have an additional enzymatic function, however some pantothenate kinase-unrelated domains of the protein could be involved in the regulation of PANK activity or in the protein-protein interaction with yet unidentified partners [49]. Although attractive, such a moonlighting role of PANK2 remains only a hypothesis, since so far it has not be supported by any experimental evidence. Alternatively, the onset of PKAN may in these 30 % of cases be due to some other, yet not identified, mutations in the genome, which act as modifiers (enhancers) of the phenotype.

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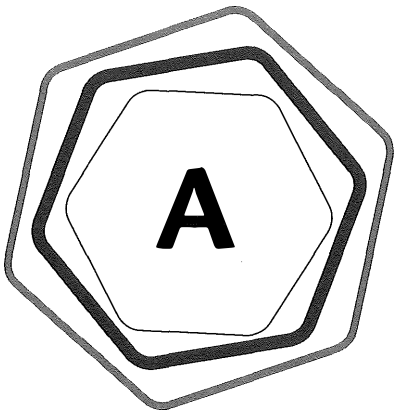

APPENDICES

Summary in Dutch

Co-enzym A (CoA) is een essentiële stof die gemaakt wordt uit vitamine B5, ATP en cysteïne. CoA wordt aangetroffen in bijna alle levende organismen. De schatting is dat CoA nodig is als cofactor voor 4% van alle bekende enzymatische reacties en dat CoA betrokken is bij wel honderd verschillende reacties van het intermediaire metabolisme. CoA reageert met carbonzuren en vormt thio-esters, en daarmee functioneert CoA als een drager en een activator van acylgroepen en het zijn de acylgroepen die deelnemen aan een groot aantal biochemische reacties. Enkele voorbeelden hiervan zijn de citroenzuurcyclus, het vetzuurmetabolisme (vetzuursynthese en de β-oxidatie) en de synthese van bepaalde aminozuren. CoA speelt dus een cruciale rol in veel chemische reacties en dit suggereert dat veranderingen in CoA-hoeveelheden of veranderingen in acyl-CoA-hoeveelheden heel veel metabole reacties kunnen beïnvloeden. Maar ondanks dat de biochemische eigenschappen van CoA al meer dan zestig jaar bekend zijn, is er relatief weinig bekend over wat nu precies de consequenties zijn van verstoringen in het CoA-metabolisme. Hiermee bedoelen we de nieuwe aanmaak van het CoA. In dit proefschrift bestuderen we in detail wat er misgaat in cellen en in hele organismen als de nieuwe aanmaak van CoA verstoord is.

Het CoA wordt gevormd uit vitamine B5 via een geconserveerde lineaire route die bestaat uit vijf opeenvolgende stappen. De eerste stap is de snelheidsbepalende stap en deze bestaat uit de fosforylering van vitamine B5 (pantothenaat). Deze reactie gaat via het enzym pantothenaatkinase (PANK). Mensen die een mutatie dragen in een van de genen (het PANK2) die coderen voor dit enzym, hebben een erfelijke recessieve autosomale neurodegeneratieve aandoening, die ook wel pantothenaat kinase geassocieerde neurodegeneratie (PKAN) wordt genoemd. PKAN-patiënten lijden onder andere aan een ernstige en progressieve vorm van verstoringen aan het bewegingsapparaat, hebben verminderde cognitieve vermogens en verliezen langzaam hun spraakvermogen. Waarom precies een mutatie in het PANK2-gen dit specifieke ziektebeeld induceert is onduidelijk. Op dit moment bestaat er geen behandeling voor deze ziekte en het is slechts mogelijk om de ernstige symptomen zo veel mogelijk te verlichten.

Recentelijk zijn er diermodellen gemaakt van deze ziekte om zo de ziekte beter te kunnen begrijpen. Het beter begrijpen van een ziekte is nodig om een therapie te kunnen ontwikkelen. Het opmerkelijke van deze studies van diermodellen is dat muizen die een mutatie dragen in het muizen-PANK2-gen, geen symptomen hebben die overeenkomen met de karakteristieke kenmerken van PKAN-patiënten. Als een alternatief voor muizenmodellen is het ook mogelijk om de fruitvlieg (Drosophila melanogaster) te gebruiken om een diermodel van een ziekte te creëren en hiermee kan vervolgens fundamentele kennis verkregen worden die nodig is om te begrijpen wat er ten grondslag ligt aan een bepaalde ziekte. Er bestaan ook zeer informatieve fruitvliegmodellen voor ziekten zoals hartfalen, mentale retardatie, de ziekte van Parkinson, de ziekte van Alzheimer, etc. De fruitvlieg heeft ook een gen dat codeert voor het enzym pantothenaatkinase en als dit gen gemuteerd is, dan vertonen de fruitvliegen een complex fenotype zoals progressieve

neurodegeneratie, een kortere levensduur, steriliteit, een verstoorde vetzuurhomeostase en toegenomen DNA-schade. Het was op het moment dat dit onderzoek startte niet duidelijk waarom een defect in de aanmaak van CoA deze specifieke combinatie aan abnormaliteiten veroorzaakt. Maar het was wel duidelijk dat het PKAN-fruitvliegmodel enkele symptomen laat zien die ook bij PKAN-patiënten waargenomen worden. Het veelzijdige fenotype van het PKAN-fruitvliegenmodel laat zien dat het inderdaad zo is dat CoA nodig is voor het in stand houden van de homeostase (homeostase is het vermogen van een organisme om het interne milieu constant te houden).

In dit proefschrift worden verschillende technieken en strategieën gebruikt die er allemaal op gericht zijn om erachter te komen wat nu precies de consequenties zijn van een verstoorde aanmaak van het CoA. De consequenties worden bestudeerd zowel op cellulair niveau als op het niveau van hele organismen. In hoofdstuk 2 en 3 laten we zien hoe gekweekte Drosophila-cellen gebruikt kunnen worden als een makkelijk toegankelijk en te manipuleren in-vitrosysteem om de PANK-enzymactiviteit uit te kunnen schakelen. We gebruiken hierbij RNA-interferentietechnologie (RNAi) om een afname van de hoeveelheden Drosophila-PANK-enzym te veroorzaken en we bestuderen vervolgens de consequenties. We tonen aan dat een normale constante nieuwe aanmaak van CoA nodig is om cellen met een normale snelheid te laten delen. Een normale onbehandelde cel gaat tijdens de celcyclus door verschillende fases heen, zoals een G1-fase, S-fase (hierin vindt replicatie van het DNA plaats), G2-fase en een mitose (celdeling). Na de celdeling worden er twee identieke dochtercellen gevormd. Hoofdstuk 3 laat zien dat een normale aanmaak van CoA nodig is om de celcyclus normaal te laten verlopen zonder vertragingen. Cellen met te lage hoeveelheden CoA hopen zich op in de G2-fase van de celcyclus en gaan vertraagd door naar de volgende fase, de mitose. Dit gaat samen met verlaagde hoeveelheden van cyclines, dit zijn eiwitten die specifiek tijdens de mitose aanwezig zijn en die een prominente rol spelen bij de normale celcyclusregulatie.

Met het in-vitrocelmodel voor PKAN is vervolgens getest of we stoffen konden vinden die de abnormaliteiten kunnen herstellen. We hebben gezocht naar stoffen die de hoeveelheden CoA herstellen en de overleving van cellen herstellen in de aanwezigheid van gereduceerde hoeveelheden PANK-eiwit. We hebben een stof gevonden die hieraan voldoet en we laten in hoofdstuk 4 zien dat er nog een andere route bestaat voor cellen om CoA te vormen, niet vanuit vitamine B5 maar vanuit deze stof, pantethine genaamd. Eerst laten we zien dat cellen en vliegen met een verstoorde functie van het PANK-enzym verlaagde hoeveelheden CoA hebben. Daarna laten we zien dat de toevoeging van pantethine in het medium van deze cellen, of het voeren van pantethine aan deze vliegen, de hoeveelheden van gereduceerd CoA weer kan herstellen. Pantethine redt daarbij ook veel andere fenotypen die geïnduceerd worden door een depletie van het PANK, zoals abnormale mitochondriën (energie producerende organellen), abnormale neuronale functies en een verkorte levensduur.

In hoofdstuk 5 bestuderen we de betrokkenheid van CoA bij de acetylering van eiwitten. Eiwitacetylering is een posttranslationele modificatie van een polypeptidemolecuul en het is bekend dat vele functies van eiwitten gemoduleerd worden door

acetylering. We laten zien dat ten minste twee belangrijke typen eiwitten een veranderde acetylering vertonen als de aanmaak van CoA verstoord is. We laten dit zien in het Drosophila-in-vitro-PKAN-model, in het fruitvlieg-PKAN-model en ook in een C. elegans-PKAN-model. De twee eiwitgroepen waar het over gaat zijn tubuline en histonen. Het is al langer bekend dat de acetylering-deacetyleringbalans van eiwitten verstoord is bij een aantal neurodegeneratieve aandoeningen. Wij laten zien dat een verlaagde acetylering van tubuline en histonen correleert met een abnormale respons in reactie op DNA-schade, afgenomen overlevingscapaciteit van cellen en abnormale bewegingscapaciteiten van het fruitvlieg-PKAN-model. Toevoeging van deacetyleringsremmers in het medium van de cellen of aan het voer van de vliegen herstelt gedeeltelijk het abnormale fenotype van deze PKAN-modellen.

In hoofdstuk 6 bekijken we nog een ander gevolg van te lage hoeveelheden CoA, namelijk veranderingen in het actine-cytoskelet. Een dynamische regulatie van actine is nodig om cellen de juiste vorm en stevigheid te geven, maar ook om de cel adequaat te kunnen laten reageren middels snelle vormveranderingen op stimuli uit de omgeving. Een verlaagde hoeveelheid CoA correleert met abnormaliteiten van het actine-cytoskelet en deze abnormaliteiten zijn geassocieerd met een verhoogde fosforylering van een geconserveerd actine modulerend eiwit, namelijk het cofiline. Ongefosforyleerd cofiline is nodig bij het snel kunnen veranderen van actine en deze activiteit wordt geremd door de fosforylering van cofiline. We suggereren dat er een interactie is tussen het CoA-metabolisme en het modificeren van actine en deze gaat via de posttranslationele modificatie (de fosforylering) van cofiline. We tonen ook aan dat in een gedifferentieerde humane neuronale cellijn remming van de PANK-activiteit resulteert in verhoogde fosforylering van cofiline. Dit correleert tevens met morfologische abnormaliteiten en de cellen kunnen geen uitlopers meer vormen in de kweekcultures. Deze resultaten laten zien dat het CoA-metabolisme ook gelinkt is aan neuronale functie door middel van het beïnvloeden van de plasticiteit van het actine-cytoskelet. Een normale regulatie van de plasticiteit van actine is nodig voor het normaal functioneren van neuronen, maar ook van vele andere cellen.

Samengevat laat dit proefschrift zien dat het CoA-metabolisme invloed heeft op een groot aantal verschillende biologisch relevante processen die tot dusver nog nooit in verband zijn gebracht met het CoA-metabolisme. Deze studies zijn nodig om het complexe fenotype te kunnen begrijpen dat ontstaat nadat het CoA-metabolisme verstoord is. Dit levert niet alleen een schat aan fundamentele inzichten op, maar is ook nodig om de pathofysiologie te kunnen begrijpen van de ziekte PKAN.

Acknowledgements

So this is it! It is funny, how many times during the course of our PhD, we (I dare to say that all PhD students share this feeling) cannot really imagine ourselves reaching this end point. Yet, we do finally make it happen.

It surely took a lot of work and determination to get to this point, but most of all, it required the involvement of many people. I believe that every experience, even the smallest one, had an impact on my work and life during these years.

First and foremost, Ody, I would like to thank you for welcoming me in your team. Your enthusiasm and optimism were really essential. For you the glass is always half full. Now, when I look back, I think that it was perfect and crucial to counteract my rather pessimistic and sometimes over critical nature. Also, thank you for your flexibility and for giving us (me and other PhD students) the freedom to steer our projects our own ways. It was a great lesson of scientific independence. Thanks especially for the last years – I think we were really "in tune" and we have reached great efficiency. So much more could have been done, if I had more time! Even more so, I'm very grateful to you for supporting my determination to finalize my PhD and to move forward to another step in my career.

Harrie, although we did not have many occasions to discuss about science and my PhD, whenever we did, I found it always very helpful and inspiring. I hope that I will keep being inspired by your enthusiasm and curiosity in science. I also enjoyed your sense of humor, which certainly has a big share in creating a specific SSCB atmosphere.

I owe my gratitude to the members of the reading committee: Prof. Erik Boddeke, Prof. Martina Schmidt and Prof. Klaas Nico Faber for accepting the task to read and evaluate the scientific quality of this thesis.

I would also like to thank our collaborators: Prof. Susan Hayflick, Prof. Suzanne Jackowski, Dr. Ellen Nollen, Dr. Dirk-Jan Reijngoud and Prof. Oliver Kayser. Their experience and scientific input were essential to give the final shape to the projects described here, and certainly helped to increase the impact of this work.

Very very special thanks to Marta and Vaishali who agreed to be my Paranimfen and to help in organizing the day of my defense. I wish I could be able to do the same for you girls.

During my PhD I was very lucky to work in a friendly atmosphere of the SSCB/SCB floor. I would like to thank all the members of the two groups for being there and for being themselves. It is you guys that create this nice working environment.

Erwin and Anil, I have shared the office with you for almost the whole PhD time. It was fun! And trust me, until the day I die I will know how to spell NEW YORK. Also Floris, Xia, Balaji, Jan and Lanjun, you were all great officemates.

A

To all the other members of SSCB, I would like to say thank you for your help and support. Willy, although we have been working together only for a short time, I consider myself truly lucky to have met you. To me you were always "the good spirit" of the lab. Thank you for some beautiful piano concerts and dance shows, which we saw together. Jannie, thank you for all your help after Willy has left the lab. I really appreciate it! Special thanks for the C. elegans work. I wouldn`t have been able to do this without your experience in handling the tinny worms. I`m grateful also to all the other SSCB members (among them especially to Annet, Serena, Maria, Jeanette, Bart, Cecile, Hette, Gerward, Marianne, Wytse, Maarten, Rob, Sarah, Yamini and Martti) and I hope I will have a chance to thank every one of you in person at the day of my defense.

Finally, there have always been some special link between the fly people`s office and the chaperon people office. Maria R., Jurre, Michel and Marianne, when I arrived in the lab, you guys, together with Floris, were like the "veterans of war" for me – always there to help and setting a good example that a PhD is actually something possible to accomplish. After that, The Girls Team: Vaishali, Ale, Melania, Melanie and Jing - truly unbelievable and unstoppable girl power! All the fun in the lab, all the dinners, drinks, and trips... Thank you for all of those and I really really hope for more! Let's always stay in touch!

Marta and Ania, it was great to share the after-lab-life with you. Especially you two, were there to talk, laugh, cry, complain, party, travel and much more. Thank you for the sweet birthday cards that were hanging above my desk until the end. I guess we will continue to share all the spicy details of our lives, even when we are far away ;-) I would like to say thank you also to everybody else, with whom I have spent my free time in Groningen (Marcin, Ewa, Luis, Emilia and Mat, Agata, and many others). Once again, I hope I will get the chance to do it in person, if we meet at my defense.

Of all the people who helped me to get where I am now, it is to my parents that I owe the most. Mamuś, Tatuś, dziękuje Wam za wszystko. To wszystko co udało mi się osiągnąć do tej pory, bez Waszego wsparcia nie byłoby możliwe. Jesteście najwspanialsi na świecie! Mimo, że dzielą nas te wstrętne kilometry, ja zawsze czułam, że stoicie za mną murem. Bartek, Tobie podziękowania za przetarcie szlaków w wyjazdach za granicę, w nauce, no i w zakładaniu rodziny. Wojtuś, po pierwsze wielkie dzięki za pomoc przy „konstruowaniu" tej książki, ale też przy innych „pracach ręcznych" – było tego trochę. Poza tym, dzięki za to, że jesteś sobą i że przecierasz zupełnie inne szlaki niż starsze rodzeństwo. Tak trzymajmy Kochani!

Last but not least, I would like to thank you, Jean-Mi. There are no words to express how grateful I am for your continuous support and love. I will just say, with you everything is simple, everything is meaningful and everything is possible.... I'm looking forwards to the years we have ahead.

Curriculum Vitae

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A

List of Publications

1. Siudeja, K., Rana, A., de Jong, J., Sibon, O.C. (Manuscript submitted) Hyperphosphorylation of actin severing protein cofilin in response to impaired Coenzyme A metabolism in Drosophila S2 cells and during neuronal differentiation in vitro

2. Siudeja, K., Srinivasan, B., Xu, L., Rana, A., de Jong, J., Nollen, E.A., Jackowski, S., Sanford, L., Hayflick, S., and Sibon, O.C. (2011). Impaired Coenzyme A metabolism affects histone and tubulin acetylation in Drosophila and human cell models of pantothenate kinase associated neurodegeneration. EMBO Mol Med. (doi: 10.1002/emmm.201100180)

3. Siudeja, K., de Jong, J., and Sibon, O.C. (2011). Studying cell cycle checkpoints using Drosophila cultured cells. Methods Mol Biol 782, 59-73.

4. Rana, A., Seinen, E., Siudeja, K., Muntendam, R., Srinivasan, B., van der Want, J.J., Hayflick, S., Reijngoud, D.J., Kayser, O., and Sibon, O.C. (2010). Pantethine rescues a Drosophila model for pantothenate kinase-associated neurodegeneration. Proc Natl Acad Sci U S A 107, 6988-6993.

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6. Olczak, T., Siudeja, K., and Olczak, M. (2006). Purification and initial characterization of a novel Porphyromonas gingivalis HmuY protein expressed in Escherichia coli and insect cells. Protein Expr Purif 49, 299-306.

7. Siudeja, K., and Olczak, T. (2005). [Mechanisms and regulation of iron and heme utilization in Gram-negative bacteria]. Postepy Biochem 51, 198-208